On the Origin of Melanoma Targeted Therapy Resistance

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1. Abstract

Melanoma skin cancer is currently the sixth most common cancer in the US and is responsible for 80% of skin cancer related deaths. Although its worldwide incidence rates have doubled over the past years, the mortality rates have remained constant, possibly due to higher public awareness and early detection. Surgical removal of early lesions remains the most effective treatment, and about 80% of melanoma patients can be cured this way. However, in approximately 20% of patients, disseminated disease develops, which results in a poor prognosis with nearly all stage IV melanoma patients dying from their disease within 5 years. Over the last decade, important steps in stage IV melanoma treatment have been made, with immunotherapy and MAPK targeted therapies showing an overall survival benefit for the first time in the history of melanoma clinical research.

MAPK targeted therapies are used in patients with a constitutively active MAPK pathway due to activating BRAF or NRAS mutations. Despite a fast response and relatively little adverse effects in a large proportion of patients, the majority of patients will develop therapeutic resistance after approximately 9 months of treatment, resulting in a relapse of tumor growth. Thus, a better understanding the molecular basis of the resistance mechanisms that develop during MAPK targeted therapy is mandatory.

The overall aim of this PhD thesis was therefore to gain insight into the evolution of targeted therapy resistance in BRAF and NRAS mutated patients treated with BRAF and MEK inhibitors, respectively, as well as to identify new resistance mechanisms and their origin.

First, to recapitulate *in vivo* tumor heterogeneity *in vitro,* we developed new protocols for the generation of cell cultures from surplus material we obtained from MAPK pathway inhibitor resistant patients who had been treated for melanoma in our clinic. We hypothesized that disease heterogeneity plays an important role in the development of resistance, and unlike other studies, we wished to work with cells that were derived from patients who became resistant to targeted inhibitors, rather than cell lines that derived resistance *in vitro*. These efforts resulted in newly developed, more efficient cell culturing protocols that are currently successfully used for our melanoma cell biobanking project (Raaijmakers et al., 2015).

Next, by whole exome sequencing of different tumors from multiple sites from single patients, we studied the evolution of targeted therapy resistance in a BRAF mutated patient treated with a BRAF inhibitor, an NRAS mutated patient treated with a MEK inhibitor and, as a control, a BRAF and NRAS wildtype patient who did not receive a specific targeted therapy but a multi-tyrosine kinase inhibitor. Through phylogenetic analysis we observed a monophyletic evolution of the different tumors from the patients treated with targeted therapy which was not observed in the patient not treated with targeted therapy. Furthermore, we identified a secondary NRAS mutation in several single cell clones generated from one particular metastasis isolated from the BRAF mutated patient, confirming that activating BRAF and NRAS mutations can co-occur in a single cell, something which was in contrast to the general belief at that time. We subsequently confirmed this finding in 5 other BRAF mutated patients treated with BRAF inhibitors. For all patients, we could find the secondary NRAS mutation only in the after-treatment samples, suggesting that this mutation either arose during treatment, or that a small amount of NRAS mutated cells was already present before treatment which did have a survival disadvantage in the non-treatment setting and therefore remained undetectable. All of the double mutated cell cultures we subsequently tested for sensitivity to different MAPK and PI3K pathway inhibitors, and a very heterogeneous response was found, indicating that the sole finding of a double mutation in a given patient cannot predict follow up therapy, but rather that additional mechanisms of resistance must also play an important role. This work was recently published in Oncotarget (Raaijmakers et al. 2016).

To further elucidate additional resistance mechanisms, in the final part of this thesis we characterized NRAS mutated, MEK inhibitor resistant cell cultures. We found that MEK162 resistance was

associated high IRS1 expression; however, it was not a cause of resistance. Nevertheless, some of the MEK resistant cells were sensitive to NT157, an IRS1 inhibitor. The exact mechanism by which NT157 acts to reduce viability in these cells will require further investigation. We also found that NT157 and MEK inhibition work antagonistically, underscoring the notion that knowing the exact working mechanism of a drug, in addition to potential interactions, is essential before rational combinations of drugs can be suggested.

In conclusion, targeted therapy is a treatment modality that provides great benefit for the survival of melanoma patients; however, further improvements must be made in alternative administration schemes and in optimal drug combinations. Because cancer in general, and melanoma in particular, is such a heterogeneous disease, resistance to targeted therapies almost seem inevitable, but understanding the exact causes of these resistance mechanisms will allow us to design better therapeutic strategies in the future.

2. Zusammenfassung

Der schwarze Hautkrebs oder das Melanom ist die derzeit sechst-häufigste Krebsart in den USA und ist für 80% der Hautkrebs–Todesfälle verantwortlich. Obwohl sich die weltweiten Inzidenzraten in den vergangenen Jahren verdoppelt haben, sind die Sterblichkeitsraten konstant geblieben, möglicherweise aufgrund des höheren öffentlichen Bewusstseins und der besseren Früherkennung. Die chirurgische Entfernung der frühen Läsionen bleibt die effektivste Behandlung, und etwa 80% der Melanom Patienten können auf diese Weise geheilt werden. Bei etwa 20% der Patienten entwickelt sich jedoch eine disseminierte Erkrankung, welche eine schlechte Prognose hat, da fast alle Stadium IV Melanom-Patienten innerhalb von 5 Jahren an ihrer Krankheit sterben. Im Laufe des letzten Jahrzehnts wurden wichtige Fortschritte in der Melanom-Behandlung erzielt. Immuntherapien und MAPK-gezielte Therapien haben zum ersten Mal in der Geschichte der klinischen Melanom-Forschung einen Gesamtüberlebensvorteil gezeigt.

MAPK-gezielte Therapien werden bei Patienten eingesetzt, deren Tumoren aufgrund der aktivierende Mutationen von BRAF oder NRAS einen konstitutiv aktiven MAPK-Signalweg haben. Trotz eines schnellen Ansprechens und relativ geringen Nebenwirkungen bei einem Grossteil der Patienten, entwickelt die Mehrheit der Patienten nach einer Behandlung von etwa 9 Monaten therapeutische Resistenz, mit erneutem Tumorwachstum. Daher interessieren wir uns für ein besseres Verständnis der molekularen Basis der Resistenzmechanismen, welche sich während der MAPK-zielgerichteten Therapie entwickeln.

Ziel dieser Doktorarbeit war es, Einblicke in die Evolution der zielgerichteten Therapie-Resistenz bei BRAF- und NRAS-mutierten Patienten zu gewinnen, welche mit BRAF- bzw. MEK-Inhibitoren behandelt wurden, sowie neue Resistenzmechanismen und deren Ursprung zu identifizieren.

Um die *in vivo* Tumor-Heterogenität *in vitro* zu erhalten, haben wir neue Protokolle entwickelt für die Erzeugung von Zellkulturen aus überschüssigem Material von MAPK-Inhibitor-resistenten Melanom Patienten, welche an unserer Klinik behandelt wurden. Wir vermuteten, dass die Heterogenität der Krankheit eine wichtige Rolle bei der Entwicklung der Resistenz spielt. Im Gegensatz zu anderen Studien wollten wir an Zellen arbeiten welche von Patienten stammen, die gegen gezielte Inhibitoren resistent wurden, anstatt Zelllinien welche *in vitro* resistent wurden. Diese effizienteren Kultivierungsprotokolle wurden als neue Methode für das Melanomzell -Biobanking publiziert (Raaijmakers et al., 2015).

Als nächstes untersuchten wir mittels whole exome Sequenzierung verschiedener Tumorlokalisationen von einzelnen Patienten die Evolution der gezielten Therapieresistenz. Dies in einem mit BRAF-Inhibitor behandelten BRAF-mutierten Patienten, einem mit MEK-Inhibitor behandelten NRAS-mutierten Patienten und als Kontrolle, in einem BRAF- und NRAS-Wildtyp-Patient, welcher keine gezielte Therapie erhielt. Durch phylogenetische Analyse beobachteten wir eine monophyletische Evolution der verschiedenen Tumoren von den Patienten, welche mit einer gezielten Therapie behandelt wurden. Dies war bei dem Patienten welcher nicht mit einer gezielten Therapie behandelt wurde nicht zu beobachten. Des Weiteren haben wir eine sekundäre NRAS-Mutation in ceiner Metastase aus dem BRAF-mutierten Patienten identifiziert, und über die Analyse einzelner Zellklone aus dieser Metastase fanden wir, im Gegensatz zu der damaligen vorherrschenden Meinung, dass die aktivierenden Mutationen von BRAF und NRAS in einer Zelle zusammen auftreten können. Wir konnten anschliessend die Existenz von aktivierende BRAF- und NRAS-Doppelmutationen in einer einzigen Zelle in 5 anderen BRAF-mutierten Patienten, die mit BRAF-Inhibitoren behandelt wurden, bestätigen. Für alle Patienten konnten wir die sekundäre NRAS-Mutation nur in den Proben nach Therapie finden, was darauf hindeutet, dass diese Mutation entweder während der Behandlung entstanden ist oder dass ein kleiner Subklon vor der Behandlung vorhanden war, welcher ohne Behandlung einen Überlebensnachteil hatte und daher klein blieb. Alle doppelt mutierten Zellkulturen haben wir anschließend auf Sensitivität gegenüber verschiedenen MAPK- und PI3K-Pathway-Inhibitoren getestet, und wir fanden ein sehr heterogenes

Therapieansprechen. Diese zeigt, dass die alleinige Entdeckung einer doppelten Mutation bei einem Patienten nicht prädiktiv für die Folgetherapie ist. Diese Arbeit wurde vor kurzem in Oncotarget publiziert (Raaijmakers et al., 2016).

Drittens wollten wir Resistenzmechanismen in NRAS-mutierten MEK-Inhibitor resistenten Zellkulturen identifizieren. Wir fanden, dass eine hohe IRS1-Expression mit der MEK162-Resistenz assoziiert war, allerdings war diese nicht die Ursache der Therapie-Resistenz. Nichtsdestotrotz waren einige der MEK-resistenten Zellen sensitiv gegenüber NT157, einem IRS1-Inhibitor. Der genaue Mechanismus mit dem NT157 wirkt, um die Viabilität dieser Zellen zu reduzieren, bedarf weiterer Untersuchungen. Wir fanden weiter, dass NT157 und MEK-Hemmung antagonistisch wirken, was wiederum zeigt, dass die Kenntnis des genauen Wirkungsmechanismus eines Medikaments essentiell ist, um rationale Kombinationen von Arzneimitteln vorgeschlagen zu können. Diese Arbeit ist derzeit in Vorbereitung für die Publikationseinreichung.

Zusammenfassend ist die gezielte Therapie eine Behandlungsmethode, welche sich bei Melanom Patienten als sehr wirksam erwiesen hat. Allerdings müssen Verbesserungen vorgenommen werden, wie gezielte Therapien verabreicht werden und in welchen rationalen Kombinationen sie verwendet werden. Weil Krebs und das Melanom insbesondere so heterogen sind, scheint die Entwicklung von Resistenzgegen gezielte Therapien fast unvermeidlich zu sein. jDas Verständnis des Ursprungs und der Natur der Resistenzmechanismen wird es uns ermöglichen, sie in zukünftigen therapeutischen Strategien besser zu vermeiden.

3. Introduction

3.1 The development and function of normal melanocytes

Early in the second month of human embryologic development, melanoblasts, which differentiate from pluripotent neural crest cells, migrate into the embryonic dermis [1]. A few weeks later, the melanoblasts migrate further into the epidermis, however, it takes until mid-pregnancy before they start producing pigment and differentiate into melanocytes [1].

Melanocytes are long-lived, dendritic cells that live for decades in the epidermis. Their function is to protect the other skin cells from UV damage by producing melanosomes containing melanin, which are taken up by adjacent keratinocytes to form a protective cap over their nucleus. Melanin in the epidermis also protects the fibroblasts in the underlying dermis. In response to UV light, keratinocytes and fibroblasts generate factors that stimulate the proliferation and synthesis of melanin by melanocytes, indicating the presence of a paracrine/autocrine communication system between the different skin cells.

3.2 The development of melanoma skin cancer

Due to their longevity, it is thought that melanocytes are prone to accumulate genomic mutations that may lead to their malignant transformation as melanoma skin cancer. Solar UV-exposure is the most important mutagen, and hence, factors related to increased susceptibility of accumulated UV damage, such as age, fair skin, solar or tanning bed UV exposure, family history (genetic predisposition, e.g. mutations in DNA repair mechanisms) and number of pigmented nevi are important risk-factors.

3.2.1 Melanoma incidence

Over the past two decades, the worldwide incidence of melanoma has doubled, making it the 5th most common cancer in men and 7th in women of the United States [2], in Switzerland it is the 4th most common cancer for both men and women [3]. Even though the incidence has doubled, the mortality rates have remained constant [4], possibly because of the higher awareness of the general public and a rise in screenings programs, leading to a higher detection of early melanomas which can be removed completely. In about 80% of the patients the melanoma is detected in an early enough state to be cured by surgery. However, for the remaining 20% (annually more than 60.000 people worldwide [4]) who present with advanced disease, the prognosis is poor, and the vast majority of patients will die from their disease. Unfortunately, without preventive measures, predictions estimate that the trend of increasing incidence will continue over the next 15 years, with consequential increases in health care costs [4].

3.2.2 Melanoma genetics

The availability of Whole Genome Sequencing or Whole Exome Sequencing (WGS and WES, respectively), allowed for the identification of novel genetic drivers of melanoma formation. Melanomas derived from sun-exposed skin sites were found to have one of the highest somatic mutation burdens compared to other malignancies, mainly due to mutations that are associated with UV radiation [5]. Due to this high mutational load, it is difficult to distinguish between driver- and bystander mutations. In a given number of melanoma samples, it is expected that driver genes contain mutations in a larger number of samples than expected by chance, however to apply this criterion in a mutational heterogeneous cancer, large sample sizes and sophisticated bioinformatic analysis are needed. Several WGS and WES studies have been performed on melanoma samples, each identifying a set of similar, prevalent mutated genes, such as BRAF, NRAS and CDKN2 and also some different genes in different mutation frequencies, depending on the analysis method used. In The Cancer Genome Atlas (TCGA), which was initiated by the National Institute of Health (NIH), melanoma samples were analyzed with 3 different methods and 13 genes were found to be significantly mutated in all three methods used, these were: BRAF (52% of 345 samples analyzed), NRAS (28%), CDKN2A (13%

mutated, 31% deleted), TP53 (5%), ARID2 (14%), IDH1 (5.7%), PPP6C (7%), PTEN (8.5%), DDX3X (6.3%), RAC1 (6.3%), MAP2K1 (5.1%), NF1 (4%) and RB1 (3.8%) (Watson et al 2015). These genes were also found at roughly the same mutation frequencies in the Broad and Yale datasets [5, 6].

BRAF, NRAS, NF1, and MAP2K1

In the majority of melanoma patients, two signal transduction pathways are often altered and activated: the MAPK (BRAF, NRAS, NF1 and MAP2K1) and/or PI3K/AKT (NRAS, NF1 and PTEN) pathways (Figure 1).

The MAPK pathway is involved in cell proliferation, differentiation and transcriptional regulation. In the MAPK pathway, BRAF is a serine-threonine protein kinase that exerts its effect mainly through the phosphorylation of MAP2K1 (MEK), which in turn exerts its effect by phosphorylating ERK. The high prevalence (approximately 50%) of BRAF mutations in all types of melanoma skin cancer, but mostly in melanomas from non-chronically sun exposed skin [7, 8], was discovered in 2002 [9]. Most mutations have consequences for the amino acid on position 600, which leads to the substitution of the amino acid valine to either glutamic acid (V600E, approximately 80-90% of cases) or lysine (V600K, approximately 5-12%) or arginine or aspartic Acid (V600R and V600D respectively, both in 5% or less of the cases) [10, 11]. All these changes result in a constitutively active BRAF and a subsequent increased phosphorylation of MEK and ERK. Under normal circumstances, a constitutively active MAPK pathway induces cellular senescence, hence a second hit is necessary and a BRAF mutation alone is not sufficient to induce malignant transformation, underscored by the observation that a BRAF mutation can also be found in benign nevi.

NRAS is a small GTP-ase that cycles between an inactive GDP bound and an active GTP bound state. NRAS mediates signals from growth factor receptors directly to downstream targets of both the MAPK and the PI3K/AKT pathways. Certain mutations in NRAS lead to a constitutively active GTPase, capable of activating downstream mediators in the absence of growth factor receptor activation. Approximately 25% of melanoma's harbor an NRAS mutation, most commonly in the form of a missense mutation that introduces an amino acid substitution at position 12, 13 or 61 [7, 12, 13]. The mutation has a slightly higher prevalence in melanomas derived from chronically sun exposed skin.

MAP2K1 or MEK1 is a serine-threonine protein kinase that is a central mediator in the MAPK pathway. Mutations often involve C>T and G>A changes, typical for UV-induced mutagenesis [14, 15]. MEK1 mutations often co-occur with BRAF or NRAS mutations [14-16].

NF1 is a tumor suppressor gene that inhibits RAS, thereby regulating the activity of both the MAPK and PI3K-AKT pathways [17, 18]. Somatic mutations within this gene have been identified in many different cancer types and germline mutations of NF1 predispose patients to several malignancies, including melanoma [18]. All described NF1 mutations [19-21], are protein-inactivating or resulting in loss of NF1 expression. In pre-clinical studies, melanoma cells with NF1 mutations were found to be resistant to BRAF inhibitors but sensitive to MEK inhibitors [22, 23].

CDKN2A and p16 loss

Somatic, as well as germline mutations, in the p16/CDK4/cyclin D1/Rb pathway can contribute to melanoma formation (Figure 1). Germline mutations in CDKN2A have been found in 35-40% of familial melanoma cases [24].

The CDKN2A locus encodes for 2 genes (p16 and p14arf) which both act as tumor suppressor genes. P16 inhibits CDK4 and CDK6 and activates the RB protein family, which results in blockage of cell cycle progression of G1 to S-phase. P14arf activates the tumor suppressor TP53.

In the TCGA melanoma dataset, CDKN2A was altered in 44% of the melanomas, 13% harboring an inactivating mutation and 31% a deletion, however some studies suggest that this is an overestimation in thin melanoma lesions, as the prevalence of CDKN2A alterations is higher in advanced melanoma tumors [25].

Surprisingly, the TCGA analysis did not identify some already well known driver mutations of melanoma, underscoring that each individual study is limited in the capability to inform completely about all the genes important in melanoma development, which is probably due to the relatively small sample size of each study for a heterogenous disease such as melanoma. Melanoma driver genes identified in other studies include PREX2 [26], GRIN2A [27], ERBB4 [28], BCL2L12 [29], SOX10 [30], MITF [30] and KIT [31].

Besides mutations in the coding region of genes, mutations in non-coding regions identified by WGS can also function as driver mutations. For example, mutations in the TERT promotor resulting in a 2 to 4 fold increase in TERT expression [32] were found to occur in 33% of primary melanomas and in 85%

of metastases [33]. Mutations in the 5'UTR region have been analyzed to a lesser extent, and until now, identified mutations are only descriptive in terms of their roles as driver mutations.

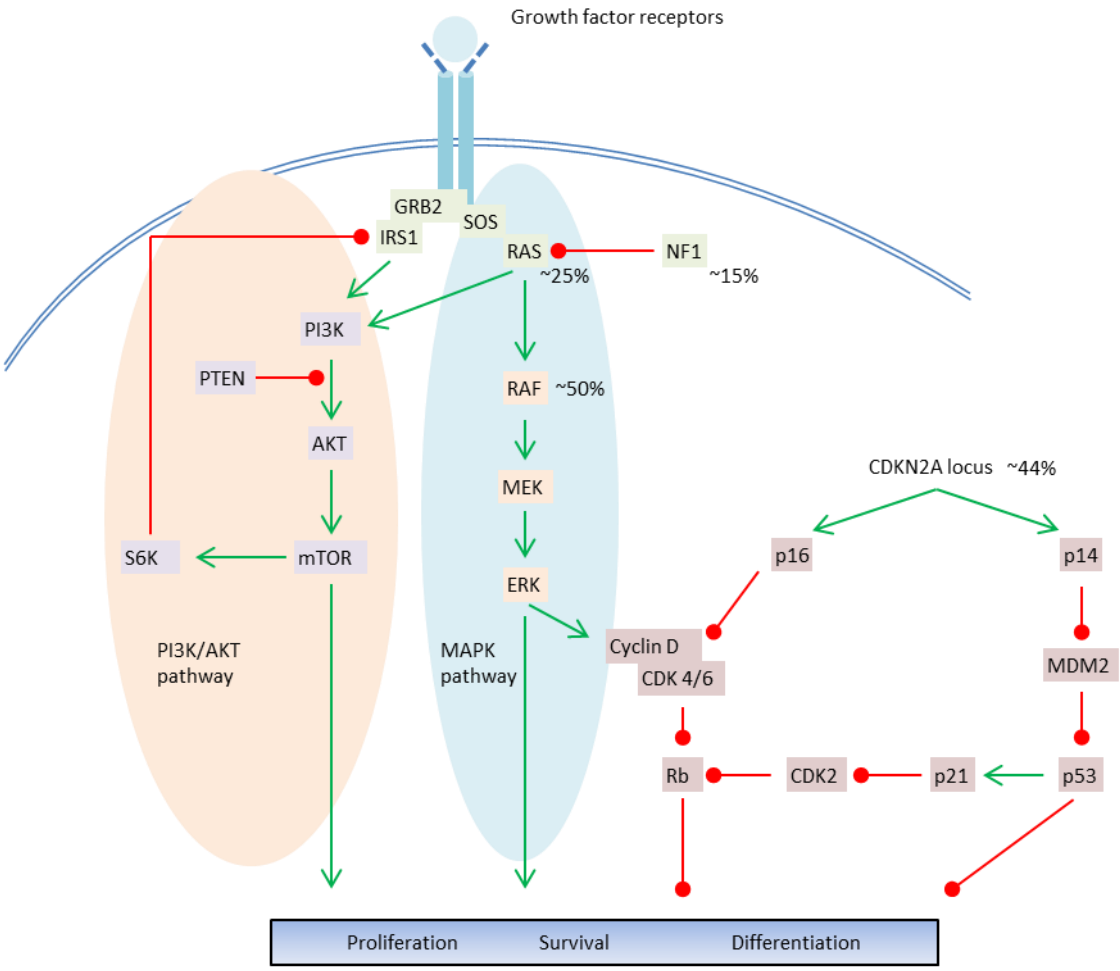

Figure 1: Signaling pathways often altered in melanoma
Green arrows indicate stimulation, red arrows indicate inhibition.
Orange box: PI3K-AKT pathway. This pathway is often hyperactivated as an escape pathway when tumors become resistant for MAPK inhibition.
Green box: MAPK pathway. Approximately 50% of melanomas harbor mutations in BRAF, 25% in NRAS and 15% in NF1 resulting in hyperactivation of the MAPK pathway, making the MAPK pathway the most altered pathway in melanoma.
Figure adapted from Wiesner et al, Pathology 2016, p113

3.3 Diagnosis of melanoma

Melanoma lesions can sometimes be very hard to distinguish from benign moles or nevi. To differentiate malignant from benign, the so-called 'ABCDE rule' [34] can be used:

A = Asymmetry
B = Border
C = Color
D = Diameter
E = Evolution

An asymmetric skin lesion with an irregular border, variegated color and a diameter of more than 6 mm is suspicious for melanoma. Additional early signs include a recent change in or development of a lesion and an "ugly duckling" sign, meaning a lesion that looks different than surrounding lesions in that patient. In a retrospective study analyzing the sensitivity and specificity of the ABCDE rule [35], the authors found that the sensitivity of the test was 97% when using one criterion and 43% when using all five criteria, indicating that not all 5 criteria must be present for the lesion to be a melanoma.

The specificity of the test is 36% when using one criterion and 100% when all 5 criteria were used, indicating that if all 5 criteria are present, it is almost certainly a melanoma.

When a melanoma is suspected, a total surgical excision is warranted with 1 to 3 mm of normal skin at the resection border whenever possible. Incisional biopsies are only indicated for large tumors or for tumors present on certain anatomical locations such as the face and ear.

A histopathologic examination is essential to confirm the diagnosis. In order for a dermatopathologist to diagnose melanoma, he or she will analyze the lesion for typical signs of cancerous uncontrolled proliferation, such as variability and abnormality in size and shape from the cells and their nuclei, prominent nucleoli and an increased amount of mitosis per phase field. The mitotic rate is the third most important prognostic factor on multivariate analysis, after the thickness of the primary tumor and ulceration (see later) [36]. Also, when melanoma is present, the melanoma cells move in all of the layers of the epidermis and in the epithelial adnexstructures. Some nests of melanoma cells will form with different shapes and sizes and with different distances to each other.
When the melanoma becomes invasive, melanoma cells can be found below the basal layer in the dermis. An important criteria is that the melanoma cells in the deeper dermis are as big and atypical as the melanoma cells on the surface.

The pathologists will also evaluate the additional prognostic factors:

- tumor thickness according to Breslow: Tumor thickness is the most important prognostic factor found by two large studies [37, 38]. The tumor thickness is measured from the surface of the skin over the tumor mass to the deepest point of invasion. Because it should be measured over the thickest part of the tumor, it is safer to evaluate it from an excisional biopsy rather than a punch biopsy as then the thickest part may be missed.
- histologic ulceration: ulceration means the absence of an intact epithelium over the melanoma lesion. In patients with tumors of the same thickness, those with ulceration have worse outcome.
- margin status
- lymphocyte infiltrates: Lymphocytes infiltrating the tumor have been associated with a better prognosis [39], however, multivariate analysis of many studies has not found a clear relationship [40].
- microsatellites: satellite lesions are considered cutaneous melanoma metastasis within a 2 cm radius of the primary tumor. Generally, they are located between the primary tumor and the draining lymph node. Macrosatellites are visible by eye, whereas microsatellites are only visible with microscopy. If the tumor is more than 3 mm thick, microsatellites are present in 30% of cases, whereas in smaller lesions this is only 5% [41].
- neural or vascular invasion

The diagnostic and prognostic information on the pathology report of the excised lesion determines clinical assessment and follow-up treatment.

3.4 Melanoma staging systems

3.4.1 TNM staging

The TNM (tumor, nodes, metastasis) staging system from the American Joint Committee on Cancer (AJCC, 7th edition) stages melanoma disease based on the most important prognostic factors.

T: primary tumor. Increased tumor thickness, increased number of mitotic figures and ulceration are associated with higher risk of relapse.
N: Presence of lymph node metastasis. Lymph node metastasis are associated with higher risk of relapse, and this is also dependent on the number and the extend of lymph node disease
M: Presence of distant metastasis.

Based on the TNM classification, patients can be divided into four stages which have shown to have prognostic value for the survival of melanoma patients:

- Stage I: These are low risk melanoma patients without regional or lymph node metastasis. Stage I is further subdivided into stage IA and stage IB, depending on tumor thickness, mitotic rate and the presence or absence of ulceration;
- Stage II: These patients also have no evidence of metastasis, however, they have a higher risk of recurrence. This stage is divided into stage IIA, stage IIB and stage IIC, depending on tumor thickness and the presence or absence of ulceration;
- Stage III: Patients have regional lymph node metastases or satellite metastases. This stage is divided into stage IIIA, stage IIIB and stage IIIC, depending on the extent of lymph node metastasis.
- Stage IV: This stage contains patients with distant metastatic disease (i.e. beyond the lymph nodes).

3.4.2 Genetic staging

Based on genetic mutations, melanomas can be categorized into BRAF mutant (52%), RAS mutant (28%), NF1 mutant (14%) and Triple-wild type subtypes (6%) [42]. In the latter, an enrichment of KIT mutation and focal amplifications and complex structural rearrangements can be observed. Depending on the mutations present, specific targeted therapies are possible.

Genetic screens, such as the DecisionDx-Melanoma, claim to accurately predict the presence of micro metastasis, independent of other prognostic factors, in patients who have been recently diagnosed with melanoma and have no visible metastatic disease yet [43]. By measuring the expression of 31 genes in a primary tumor, it can classify patients in high-risk or low-risk categories. Combined use of the genetic screen and the TNM staging system is more precise compared to the genetic screen alone [44].

3.5 Therapeutic options for melanoma

3.5.1 Surgery

Surgical intervention is important in the initial removal of the melanoma (Figure 2). Luckily, the majority of melanoma patients are diagnosed in the low risk category, i.e. patients with TI or IIA (and N0 M0) disease (tumor thickness below 2 mm with ulceration or below 4 mm without ulceration), and in this category surgical excision is usually curative.
However, surgery also has a place in:

- Regional lymph nodes

If there is a risk of regional lymph node metastasis, lymphatic mapping (based on the observation that melanomas on specific body sites have specific patterns of lymphatic spread) and sentinel lymph node biopsy are performed. When positive nodes are found, this is followed by complete lymphadenectomy. However, the effect on survival is uncertain [45-50].

- Local recurrences
- Satellite metastases
- Distant metastatic disease

3.5.2 Chemotherapeutic drugs

As chemotherapeutics have not improved overall survival and the response rates are low (less than 20%) [51], these drugs are limited to patients who cannot be treated with immunotherapy checkpoint inhibitors or targeted therapy (see below).

3.5.3 Radiation therapy

Radiation therapy is mainly restricted to palliative care for localized symptomatic disease. Caution should be paid with radiation in combination with BRAF inhibitors (see below). Significant toxicity can be prevented if treatment with BRAF inhibitor is interrupted at least three days before and after fractionated radiotherapy and one day before and after stereotactic radiotherapy [52].

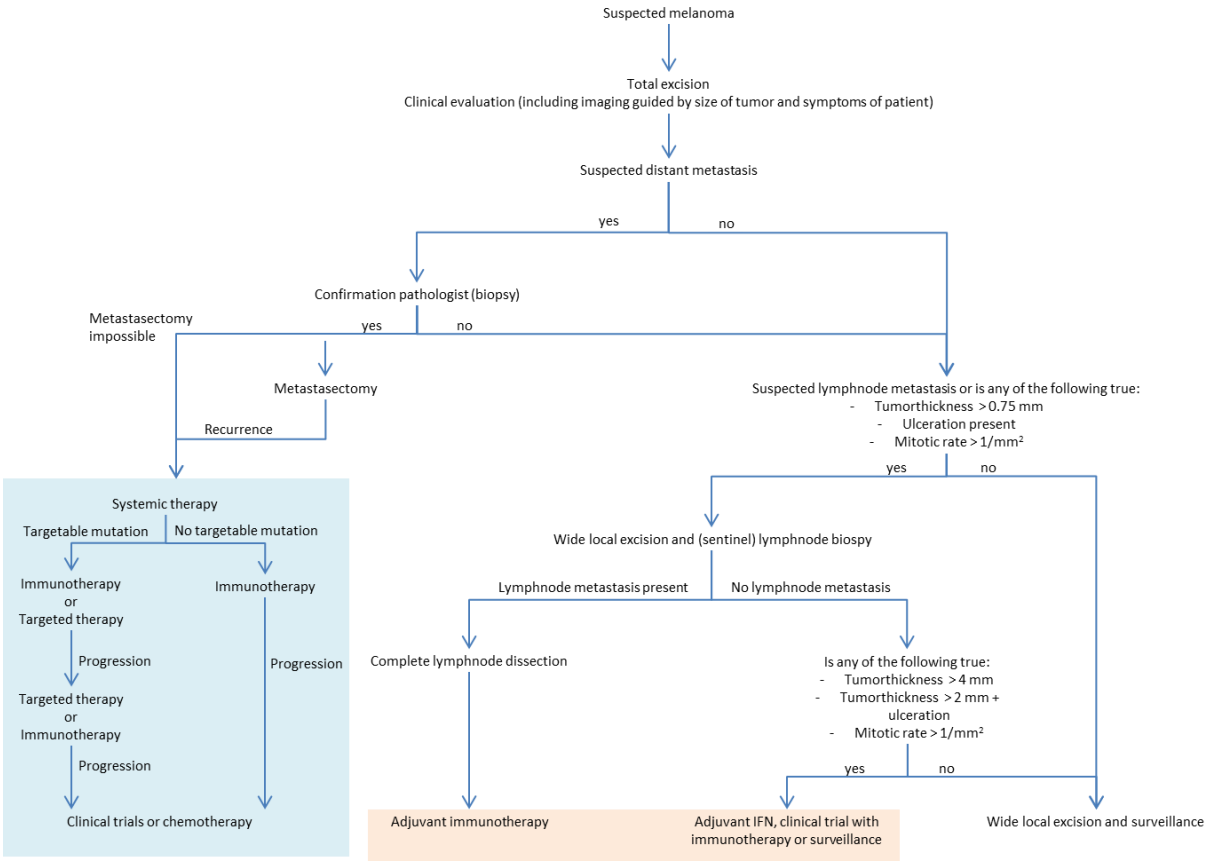

Figure 2: Clinical Decision Making Algorithm Melanoma
Clinical assesment after excision of a suspected melanoma is guided via prognostic factors on which the TNM classification is based.
Blue box: Systemic therapy. When a targetable mutation (e.g. BRAF, NRAS, KIT) is present, no data are available whether immunotherapy or targeted therapy as the initial therapy should be given (clinical trial EA6134). When a patient has progressive disease on the initial therapy, the other treatment (targeted therapy or immunotherapy) can be given. For immunotherapy combination therapy with anti-PD1 and anti-CTLA4 is preferred in healthy patients with no comorbidities.
Orange box: Adjuvant therapy. Clinical trials with adjuvant anti-PD1 immunotherapy are currently undertaken, alternatively anti-CTLA4 has been approved in patients with node positive disease. For patients with node negative disease, no data on the benefit of adjuvant immunotherapy are available; however adjuvant IFN has been demonstrated beneficial.
Figure adapted from Graphic 105384 and Graphic 109603 from Uptodate.com

3.5.4 Immunotherapy

Immunotherapy is based on the notion of using the immune system's ability to destroy "non-self" invaders for the eradication of "self" cancer cells. Although these "self" cancer cells originate from the patient, they are likely to express cancer-specific antigens, such as products from mutated genes or products from genes expressed specifically in cancer cells (e.g. cancer-testis-antigens, these genes are normally only expressed in the testis, where the blood-testis barrier prevents the entrance of immune cells [53], but can be re-expressed in tumor cells, including melanoma, upon dedifferentiation of the tumor cells), or differentiation antigens from the tumors tissue of origin (e.g. melanocytic differentiation antigens) [54], which can all serve as antigens which the immune cells can use to recognize tumor cells.

Anti-PD1 and anti-CTLA4 inihibitors both work by blocking an inhibitory interaction (CTLA4 on T-cells with B7 on dendritic cells in the activation phase of the immune response, and PD-1 on T-cells with PD-1 ligand on tumor cells in the effector phase of the immune response, respectively) which normally functions to stop T-cell activation, as a safety mechanism to prevent the destruction of “self” tissue. However, by blocking this inhibitory interaction, T-cells are stimulated to eradicate tumor cells.
In patients with metastatic disease who receive immunotherapy, a combination with anti-PD1 antibodies (pembrolizumab, nivolumab) with anti-CTLA4 antibodies (ipilimumab) is preferred, as this increases response rate and tumor shrinkage compared to either treatment alone [55]. However, serious adverse effects with required discontinuation of treatment were more frequent in the combination therapy than in anti-CTLA4 or anti-PD1 monotherapy.
In the phase III KEYNOTE-006 trial, anti-CTLA4 monotherapy was directly compared to anti-PD1 monotherapy, showing an increased progression free survival and overall survival in the anti-PD1 treated patients in all patients subsets (with and without BRAF mutation, with and without prior targeted therapy), except for patients without expression of PD-L1 on their tumors, in which no difference between anti-PD1 and anti-CTLA4 treatment was found [56].
Patients who have been treated before with ipilimumab without benefit from treatment or, in case of a BRAF mutant tumor, with BRAF inhibitors and progressed under these inhibitors, can still benefit from anti-PD1 treatment, independent of prior treatment response [57]. Considering the results from 4 independent clinical trials, BRAF WT and BRAF mutated patients had the same response rate (approximately 33%) and same response duration (approximately 12 months) on anti-PD1 therapy [58]
After 3 years of treatment, a plateau is observed in the survival curves of anti-CTLA4 treated patients, resulting in a durable response in approximately 20% of patients, even after stopping treatment [59].
In the case of a BRAF mutated patient for whom both the option of targeted therapy or immunotherapy is available, choice is dependent on performance status of the patient, due to lack of available data yet (randomized clinical trial EA6134 currently taking place). Patients with worse performance status are suggested to start with targeted therapy, due to the faster response (rapid tumor regression) and higher response rate and as it is relative well-tolerated by patients.
Adjuvant immunotherapy is available for patients who do not have clinical evidence of distant metastases, but do have a high risk tumor, based on prognostic factors such as tumor thickness, presence of ulceration and presence of lymph node metastases. Interferons (IFNs) are pleiotropic cytokines which induce the synthesis of hundreds of different proteins by activating the JAK-STAT pathway, they have an important role in inflammation and immune activation, and they have been shown to have anti-proliferative activities [60]. Adjuvant therapy with IFN alpha has been demonstrated to significantly increase disease-free survival (DFS) [61-64] and in some studies overall survival (OS) [65-67]. Therefore, high risk, node negative (TIIB or IIC) patients (tumor thickness higher than 4 mm or higher than 2 mm with ulceration), who are at increased risk for disease recurrence, as well as patients with microscopic disease smaller than 1 mm in a single lymph node will be monitored and might benefit from adjuvant high-dose IFN alpha. As these patients were excluded from the EORTC 18071 trial, no data on anti-CTLA4 in the adjuvant setting in these patients is available.
In the case of macroscopic lymph node involvement, multiple lymph nodes or a single lymph node with more than 1 mm of microscopic disease, patients can be enrolled in a clinical trial which evaluates the effect of anti-PD1 treatment in the adjuvant setting, or when clinical trial participation is not possible, adjuvant high dose anti-CTLA4 treatment (10 mg/kg) has been approved for this patient group. Participation in the clinical trial setting with anti-PD1 is preferred, although no data are yet available in the adjuvant setting, in the metastatic setting these drugs (alone or in combination with anti-CTLA4 treatment) are more effective than single-agent anti-CTLA4 treatment. Trial Eastern Cooperative Oncology Group [ECOG] E1609, NCT01274338 is currently directly comparing high dose CTLA4, normal dose CTLA4 or IFNa in this patient group, for which the results are expected in 2018, however, based on the magnitude of overall survival benefit of anti-CTLA4 vs placebo, anti-CTLA4 treatment is preferred until then [68].
Compared to cytotoxic or targeted therapy (see next session), there are some differences in the pattern of response with immunotherapy. The response on treatment may be delayed, and the disease may even worsen at onset, before stabilizing or regressing. Furthermore, patients who do not respond on first sight may have clinically relevant periods of stable disease [69].
Last but not least, toxicities with immunotherapy are significant, with the most common serious adverse events being enterocolitis, hepatitis, dermatitis and endocrinopathies, but side-effects in other organs also have been described . For a more detailed review of the various immunotherapy options available to melanoma patients, please see a review that we published a few years ago (Raaijmakers et al. 2013, Chapter 8 in this booklet).

3.5.5 Targeted therapy

Targeted therapy aims to specifically target cancer cells, usually by inhibiting one of the pro-survival pathways on which the cancer cells have shown to be reliant. Approximately 90% of melanomas (BRAF, NRAS or NF1 mutated) are dependent on an overactive MAPK pathway. Specific BRAF inhibitors (only targeting mutated BRAF), general MEK inhibitors and, recently, general ERK inhibitors have been shown to be beneficial in melanoma patients with metastatic disease .
Because in approximately 40 to 60% of melanoma patients the presence of an activating BRAF mutation is found, tumors of patients with advanced melanoma should be tested for such mutations [70-72]. In such cases, treatment with a BRAF inhibitor, preferably in combination with a MEK inhibitor, (dabrafenib with trametinib, or vemurafenib with cobimetinib) are preferred as they show improved progression free and overall survival compared to BRAF inhibition alone [73]. Currently, for patients with BRAF mutated melanoma without metastases but with high metastases risk, trials are performed to evaluate targeted (combination) therapy in the adjuvant setting (vemurafenib; trial NCT01667419, dabrafenib plus trametinib; trial NCT01682083).
The median survival for patients receiving the BRAF and MEK inhibitor combination is approximately two years. However, 9 to 12 months after initiation of treatment, patients may develop progressive disease due to resistance of the tumor cells to therapy.
Common toxicities associated with BRAF inhibition (reported in 15% or more of patients) include cutaneous side effects (squamous cell carcinomas, photosensitivity, skin rash, alopecia), arthralgia's, headache, weakness and/or fatigue.
When a BRAF inhibitor is given in combination with a MEK inhibitor, the development of squamous cell carcinoma is reduced [74]. Studies indicated that the development of squamous cell carcinomas is due to a paradoxical activation of the MAPK pathway in non-mutated cells upon treatment with BRAF inhibitor alone [75], and that the addition of a MEK inhibitor can block this paradoxical activation [74].
Single agent MEK inhibitor treatment may have a role in BRAF wildtype but NRAS mutated melanoma patients, where it was shown in a phase III (comparing binimetinib with dacarbazine in NRAS mutant melanoma) trial to significantly increase progression free survival but with no difference in overall survival at interim analysis [76]. If NRAS mutated patients also have a p16 loss, combination therapy of a MEK inhibitor together with a CDK4/6 inhibitor has been found to have significant anti-tumor responses in two thirds of patients [77].
KIT mutations are present in 25% of mucosal and acral melanomas, approximately 30% of these patients respond to several KIT inhibitors, such as imatinib [78-80], sunitinib [81], sorafenib [82] and nilotinib [83].

3.6 The development of targeted therapy resistance

Unfortunately, most patients receiving targeted therapy will develop therapeutic resistance after some months of successful treatment. Over the past years, research has focused on tumor cell resistance mechanisms mainly in BRAF mutant patients treated with a BRAF inhibitor such as vemurafenib. Reactivation of the MAPK pathway is an important resistance mechanism that was seen in nearly all BRAF inhibitor resistant tumors from the BRIM-2 vemurafenib phase II trial [84]. The same was found by Shi et al, in a study where they sequenced the genomes of 71 resistant post-treatment samples and found in 70% of the cases MAPK reactivation as a mechanism of resistance, either by an additional NRAS or KRAS mutation, mutant BRAF amplification or BRAF alternative splicing, or by CDKN2A loss [16]. In 22% of the cases they found several genetic alterations in regulators of the PI3K-AKT pathway, which were proven to be functional in inducing resistance by overexpression and knockdown studies. Furthermore, by analyzing several metastases from the same patient, they could identify at least 5 different resistance mechanisms in that patient, indicating that the selection of follow-up therapy should not be planned on one post-treatment biopsy only.

The finding that multiple resistance mechanisms co-exist in different metastases from the same patient, raises the question whether small populations of different resistant subclones were already present before treatment which become selected during treatment due to the survival advantage they have over sensitive tumor cells, or whether resistant tumors result from prior sensitive cells which have adapted to the treatment. A recent study in EGFR inhibitor resistance in lung cancer found that both processes can happen and that these different processes might provide an explanation as to why some patients progress quickly under treatment whereas others have a much longer response

duration [85]. Furthermore, they noticed that, prior to the appearance of a resistant mutation in their cell line under EGFR inhibitor treatment, a state of drug tolerance was present which was characterized by the ability to evade drug-induced apoptosis and by a mesenchymal transcription profile and that these molecular features of the drug-tolerant state - which impact general drug sensitivity - may be maintained even after acquisition of the resistant mutation for a specific drug [85].

Also in melanoma it has been acknowledged for some years that besides the permanent genetic changes that lead to drug resistance, also possibly transient or reversible non-genetic stages of resistance can be found. A drug tolerant state in melanoma [86], characterized by a reduced expression of melanocytic lineage specific markers and an increased expression of stemness and Epithelial-To-Mesenchymal (EMT) markers and of genes involved in epigenetic remodeling can be found after a relatively short exposure to targeted therapy. For instance, the enzyme KDM5B, which is a marker for a slow cycling phenotype with increased drug tolerance [87, 88], was upregulated in the stress response after the initiation of therapy. An early adaptive response upon BRAF inhibitor therapy can occur as soon as 24 hours after initiation of therapy [89]. Upon treatment, cells change morphology to a flattened and enlarged phenotype and increased senescence associated SA-β-Gal activity, H3K9me3-positive heterochromatic foci and PML bodies [90]. Also other stressors, such as hypoxia or low glucose can induce a similar stress response. Interestingly, when cells that exhibit such a stress response are exposed to various drugs, they are more resistant than their non-stressed equivalents, pointing towards a general drug tolerant state.

Besides cell autonomous processes that generate resistance, such as genetic mutations or epigenetic alterations that come from the cell itself, processes outside the cell (cell non-autonomous processes) can also contribute to resistance. Different tumor subclones can collaborate with each other or with other cells in their tumor micro-environment in order to withstand treatment. For instance, treatment with a MAPK pathway inhibitor induces sensitive cells to secrete a treatment induced secretome (TIS), which components have the ability to directly or indirectly activate the PI3K-AKT pathway in resistant cells, which thereby can overcome their semi-quiescent state and become more proliferative while staying resistant [91]. Combination therapy of MAPK inhibitors with PI3K-AKT pathway inhibitors could prevent the therapy induced accelerated expansion of resistant cells in mice [91]. On the opposite, MAPK pathway inhibitor treatment also induces sensitive cells to express danger signals which incite an immune response, here the resistant cells have a dampening effect on that, thereby preventing an immune attack on the sensitive cells [92].
Tumor cells can also collaborate with non-tumor cells in their micro-environment, such as fibroblasts. Hirata et al showed that fibroblasts increase their expression of PDGFRa in response to treatment with BRAF inhibitors, due to the paradoxical activation of the MAPK pathway in non-mutated cells from BRAF inhibitors. This results in the production of a denser collagen fibril matrix, with alterations in integrin organization and FAK signaling, which directly provides a safe haven for melanoma cells to withstand BRAF inhibitor therapy [93]. Interestingly, when these melanoma cells are taken out of the altered matrix, they are sensitive again to BRAF inhibition.

In order to find new therapeutic strategies to overcome melanoma drug resistance, it is important to have a good overview of all the strategies melanoma cells use to overcome drug treatment. In the past years, we have tried to contribute a little piece of knowledge and insight in the huge melanoma puzzle, with the hopes that in the future, metastatic melanoma can be cured or can become a chronic disease which can be managed over a long period.

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4. Aims of the Thesis

The general aim of this thesis was to gain insight into the development of MAPK pathway inhibitor resistance in advanced melanoma.

- Finding new biobanking methods in order to improve the successrate of establishing cell cultures and to recapitulate the *in vivo* tumor heterogeneity in an *in vitro* setting
- Investigate how therapeutic resistance develops within single patients
- Identify (new) resistance mechanism and investigate alternative strategies to target MAPK-inhibitor resistant melanoma cells

5. Contribution to the different manuscripts and reviews

Papers covered in this thesis

Paper 1: A new live-cell biobank workflow efficiently recovers heterogeneous melanoma cells from native biopsies

For this paper I developed and experimentally validated the different culture techniques for efficient retrieval and generation of *in vitro* melanoma cell cultures out of native patient biopsies. I described our findings in this paper for which I am first author.

Paper 2: Co-existence of BRAF and NRAS driver mutations in the same melanoma cells results in heterogeneity of targeted therapy resistance

For this paper I performed all the *in vitro* melanoma experiments, as well as Sanger sequencing of cell cultures and histology blocks and preparation of the cell and/or tumor DNA for the whole exome sequencing, ultra-deep seqencing and digital PCR part. I described our findings in this paper for which I am first author.

Manuscript 1 in preparation: IRS1 expression is correlated with, but it is not a direct mechanism of MEK162 resistance in NRAS mutated melanoma

For this manuscript I assisted the pathology with the patients' autopsy, selected the tumors and generated the cell cultures from these tumors, performed all the subsequent *in vitro* experiments and generated the DNA and RNA for the whole exome sequencing and RNA sequencing experiments. I described our findings in this paper for which I am first author.

Review 1: Melanoma immunotherapy: historical precedents, recent successes and future prospects

For this review I wrote most of the sections and I coordinated the overall process. I am first author of this review.

Review 2: Metastatic melanoma moves on: translational science in the era of personalized medicine

For this review I wrote the sections on melanoma resistance (genomic, transcriptional and non-cell autonomous) for targeted MAPK inhibitors. For this review I am a co-author.

Papers not covered in this thesis

Paper 3: Hypoxia contributes to melanoma heterogeneity by triggering HIF1a-dependent phenotype switching

For this paper I performed *in vitro* growth assays for different cell cultures. For this manuscript I am a co-author.

Paper 4: Methylation-dependent SOX9 expression mediates invasion in human melanoma cells and is a negative prognostic factor in advanced melanoma

For this paper I assisted in *in vitro* experiments. For this manuscript I am a co-author.

6. Paper 1: A new live-cell biobank workflow efficiently recovers heterogeneous melanoma cells from native biopsies

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A new live-cell biobank workflow efficiently recovers heterogeneous melanoma cells from native biopsies

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Abstract: Fibroblast contamination can make establishing primary melanoma cell cultures from native biopsies a major challenge, due to fibroblasts overgrowing the melanoma cells. Standard protocols therefore enrich for highly proliferative melanoma cells that grow well *in vitro* but may not represent the full range of *in vivo* tumor heterogeneity. Here we apply conditional methods that more effectively retrieve melanoma cells by differential trypsinization or by inducing fibroblast senescence through contact inhibition, serum starvation or deprivation of adhesion. Simple mixing experiments of melanoma and fibroblast cells demonstrated the efficacy of the new protocols in retrieving slow-growing melanoma cells. Applying our protocols to 20 cultures that had failed to grow by conventional methods, we could retrieve 12 (60%) validated melanoma cell cultures. Further application of the protocols in the live-cell biobank of 124 early passage cultures significantly improved recovery rates from 13% using standard protocols to 70% overall for the new workflow.

Key words: melanoma – cell culture – biobank – fibroblast overgrowth

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Introduction

Fibroblasts can complicate the establishment of primary melanoma cell cultures from native biopsies (1,2). Thus, highly proliferative melanoma cells that grow well *in vitro* may be over-represented in biobanks using standard culturing procedures and may not recapitulate the full range of *in vivo* tumor heterogeneity (3–5). Various cellular features that differ between fibroblasts and melanoma cells can be used to retrieve melanoma cells from mixed cultures. To avoid the loss of valuable patient samples, we have tested different methods to enrich for melanoma cell cultures out of fibroblast-rich biopsies.

We tested two different methods that induce fibroblast senescence while preserving melanoma cell proliferation:

1. Fibroblasts stop proliferating when they reach a certain density directly related to the confluency of the cell culture and inversely related to the foetal calf serum (FCS) concentration of the culture medium (6). FCS contains growth factors that are essential for fibroblast proliferation but not for metastatic melanoma proliferation (6,7). In addition, fibroblasts secrete factors that stimulate melanoma cell proliferation (8,9). Combining these findings, we cultured fibroblast-rich biopsies for an extended time without splitting and changed the medium only once every 2 weeks. In this way, fibroblasts stopped proliferating due to contact inhibition and serum deprivation, whereas melanoma cells could continue proliferating.
2. In a second approach, we cultured cells on ultra-low-binding plates. Fibroblasts that cannot adhere arrest in the G0/G1 phase of the cell cycle (10,11), whereas melanoma cells do not undergo adhesion induced apoptosis (anoikis) and can even proliferate under non-adhesive conditions (12).

Finally, to generate pure melanoma cell cultures, we combined two different techniques based on the difference in adhesive properties between the two cell types. Fibroblasts are adherent cells that are relatively difficult to trypsinize, especially when they have been cultured for an extended time without splitting and have generated extracellular matrix (13). Melanoma cells on the other hand detach relatively easily upon trypsinization, even when they grow in a layer on top of the fibroblasts. A further selection can be made using the difference of adhesion time between fibroblasts and melanoma cells. Fibroblasts adhere more quickly to the culture dish's surface (i.e. around 30 min after seeding) than melanoma cells, which may require more than an hour after seeding to properly adhere (data not shown).

Finally, our simple mixing experiments of melanoma and fibroblast cells demonstrated the efficacy of the new protocols in retrieving slow-growing melanoma cells. Applying our protocols to 20 cultures that had failed to grow by conventional methods, we could retrieve 12 (60%) validated melanoma cell cultures. Further standardization of the protocols in the live-cell biobank of 124 early passage cultures significantly improved recovery rates from an initial 13% to a 70% overall success rate with the new methods.

Material and methods

Standard cell culture

Cell cultures were obtained from patient biopsies of cutaneous melanoma and distant melanoma metastases after informed consent according to ethical approval numbers 647 and 800 and following the Declaration of Helsinki on human rights. Tumor material was processed as described in Widmer et al. (3). Briefly, tumor material was divided into small pieces and digested with 2.4 U/ml dispase (Roche, Basel, Switzerland), followed by a digestion with 62.5 U/ml collagenase (Sigma, St. Louis, MO, USA), so that the final suspension consisted of separated cells. This suspension was cultured in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 5 mM L-glutamine (Biochrom, Berlin,

Germany), 1 mM sodium pyruvate (Gibco, Carlsbad, CA, USA) and 10% FCS (Gibco) at 37°C and 5% CO_2. Cultures were split when they were approximately 90% confluent, and medium was refreshed twice per week.

No splitting, limited culture medium change

Cell cultures were handled as in the standard protocol above, but without splitting and with medium changes only once every 2 weeks at 37°C in 5% CO_2 atmosphere.

Short trypsinization and selective adherence method

If nests of melanoma cells grew visibly on top of fibroblasts, the cell cultures were rinsed two times with PBS and trypsinized long enough for only the cells on the top layer to detach, which is approximately 1 min at room temperature. The detached cells were collected and seeded in supplemented RPMI1640 with 10% FCS and incubated for 30 min at 37°C in 5% CO_2 atmosphere. After 30 min, the supernatant was transferred to another dish and cultivated further at 37°C in 5% CO_2. The protocol was repeated if there were still some fibroblasts left at the end of the procedure.

Ultra-low-binding plates

Cells were plated in 6-well low-binding plates (catalogue number 3471, Falcon, Corning, NY, USA) for 5 weeks. Medium was carefully replaced every week to prevent damage of the cellular aggregates. After 5 weeks, aggregates were plated in uncoated and untreated polystyrene 6-well culture plates (Falcon), in order for cells to attach and grow out of the aggregates.

Spiking experiment

Independent cultures of 3^10^5 fibroblasts, derived from biopsies of normal skin, were spiked with six melanoma cells from any of the fast-growing cell lines M000921, M130429 or M130427, which carry the $BRAF^{V600E}$, the $NRAS^{Q61R}$ and the $NRAS^{Q61R}$ mutations, respectively. Six melanoma cells from the slow-growing cell line M130219, which carries the $NRAS^{Q61R}$ mutation, were also added to a separate culture of 3 10^5 fibroblasts. An average of four counts with the Neubauer chamber was used to prepare the appropriate cell concentrations. For each culture, four different methods to retrieve the melanoma cells were compared in parallel: the 'standard culture' protocol, the 'no splitting, limited culture medium change' approach, the 'short trypsinization and selective adherence method' and the ultra-low-binding plates. After 3–6 weeks, cultures were tested for $BRAF^{V600E}$ or the $NRAS^{Q61R}$ mutation by Sanger sequencing.

Retrieval of melanoma cells from previously identified fibroblast cultures

Cultures were isolated from melanoma biopsies with known oncogenic driver mutations (based on Sanger sequencing of the tumor) but from which only non-mutated, likely fibroblast cultures grew (based on morphology and Sanger sequencing) with the standard protocol. To retrieve the melanoma cells, these cultures were subjected to the 'no splitting, limited culture medium change' approach followed by the 'short trypsinization and selective adherence' method.

Mutation analysis melanoma cell cultures

DNA was isolated using a buccal swab (Raucotupf, Lohmann & Rauscher, Rengsdorf, Germany), in combination with the QiAmp DNA blood mini kit (Qiagen, Hilden, Germany). The DNA was sequenced by Sanger sequencing.

Primer sequences were as follows: BRAF: forward 5'CTAAGAGGAAAGATGAAGTACTATG and reverse 5'CTAGTAACTCAGCAGCATCTCAG; NRAS: forward 5'GATAGGCAGAAA

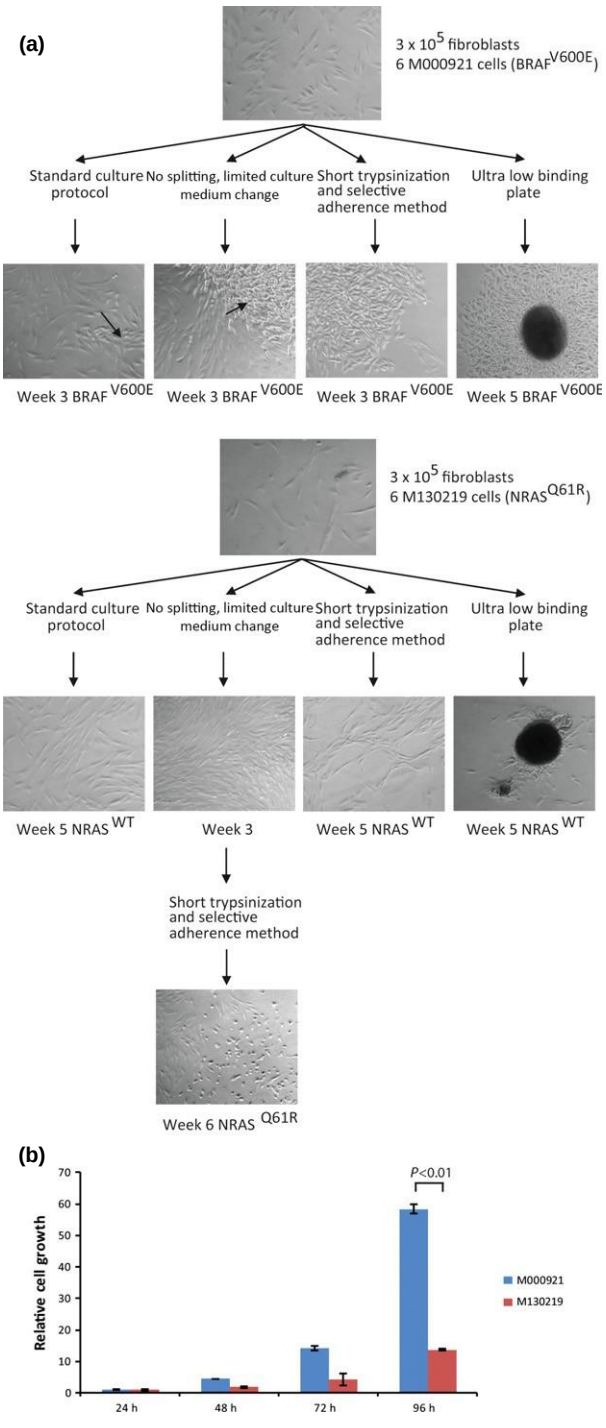

Figure 1. Schematic view of spike experiment results. (a) 3 9 10^5 fibroblasts were spiked with six melanoma cells either from the fast-growing cell lines M000921 (representative pictures shown), M130429 (not shown) or M130427 (not shown), or from the slow-growing cell line M130219. Each spiked culture was applied to four different conditions for parallel comparison of methods. For the cultures spiked with M000921 cells, all methods resulted in retrieval of the melanoma cells, as observed by Sanger sequencing of the $BRAF^{V600E}$ mutation. However, the M130219 cells were only retrieved with the 'no splitting, limited culture medium change' protocol for the first weeks and a subsequent 'short trypsinization and selective adherence' method. Arrows indicate melanoma cells that grow in a layer on top of the fibroblasts. (b) BrdU assay showing that M000921 is a proliferative cell line and M130219 grows relatively slowly. *P*-values were calculated by Student's *t*-test of four independent replicates.

TGGGCTTGA and reverse 5⁰ATCATCCTTTCAGAGAAAATAATGC.

BrdU assay

The BrdU assay was performed using the BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

Statistics

Data were analysed for statistical significance with a chi-square test and a *Z*-test using a *P*-value of <0.01 at http://www.socscistatistics.com/. The BrdU assay was analysed using a *T*-test.

Results

Slow-growing melanoma cells require alternative culturing methods

When the fast-growing BRAF- or NRAS-mutated melanoma cell lines were mixed with fibroblasts, cells with the oncogenic driver mutation could be retrieved with all methods tested, notably also with the standard culture protocol (Fig. 1). This was repeated with three different cell lines. However, slow-growing NRAS-mutated melanoma cells could only be retrieved with the 'no splitting, limited culture medium change' method, followed by the 'short trypsinization and selective adherence' method.

Alternative methods improve the recovery of melanoma cells from impure cultures

Twenty cultures from BRAF- or NRAS-mutated melanoma biopsies, which had been identified as cultures overgrown by fibroblasts based on the recovery of only the WT oncogene allele upon Sanger sequencing of the cultures, were subjected to the 'no splitting, limited culture medium change' method, followed by one or more rounds of the 'short trypsinization and selective adherence' method. In this way, we were able to retrieve 11 (55%) of 20 melanoma cultures that had previously failed the standard culturing procedures (Table 1). A subset of the 9 WT cultures that had failed the standard protocols as well as our alternative methods was subsequently seeded on low-binding plates. With this method, we were able to retrieve 1 additional cell line of 7 (14%),

Table 1. Isolation of melanoma cells from previously wild-type cultures

Cell culture	Patient mutation status	Biobank mutation status	Mutation status after the no splitting, limited culture medium change method, followed by the short trypsinization and selective adherence method	Low binding plate
M121224	$BRAF^{V600E}$	WT	$BRAF^{V600E}$, $NRAS^{Q61K}$	
M130117	$NRAS^{Q61R}$	WT	$NRAS^{Q61R}$	
M121225	$BRAF^{V600E}$	WT	$BRAF^{V600E}$, $NRAS^{Q61K}$	
M130434	$BRAF^{V600E}$	WT	$BRAF^{V600E}$	
M130610	$BRAF^{V600K}$	WT	$BRAF^{V600K}$	
M121102	$NRAS^{Q61L}$	WT	$NRAS^{Q61L}$	
M140211	$NRAS^{Q61L}$	WT	$NRAS^{Q61L}$	
M140307	$BRAF^{V600E}$	WT	$BRAF^{V600E}$	
M140906	$BRAF^{V600E}$	WT	$BRAF^{V600E}$/ $NRAS^{Q61K}$	
M140902	$NRAS^{Q61K}$ $BRAF^{V600K}$	WT	$NRAS^{Q61K}$ $BRAF^{V600K}$	
M140307	$BRAF^{V600E}$	WT	WT	
M130430	$BRAF^{V600E}$	WT	WT	$BRAF^{V600E}$
M120710	$BRAF^{V600E}$	WT	WT	WT
M120522	$NRAS^{Q61R}$	WT	WT	WT
M130528	$BRAF^{V600E}$	WT	WT	WT
M140523	$BRAF^{V600E}$	WT	WT	WT
M130514	$BRAF^{V600E}$	WT	WT	Failure to grow
M140206	$BRAF^{V600K}$	WT	WT	Failure to grow
M140224	$BRAF^{V600E}$	WT	WT	Not tested
M140506		WT		Not tested

Twenty cultures from BRAF- or NRAS-mutated tumors that had been overgrown by fibroblasts in standard culturing conditions (as shown by their WT mutation status) were subjected to the 'no splitting, limited culture medium change' method, followed by the 'short trypsinization and selective adherence' method. A subset of seven cultures that still tested WT after this method was seeded onto low-binding plates. The BRAF or NRAS mutation status was checked by Sanger sequencing subsequent to culturing with the new protocols.

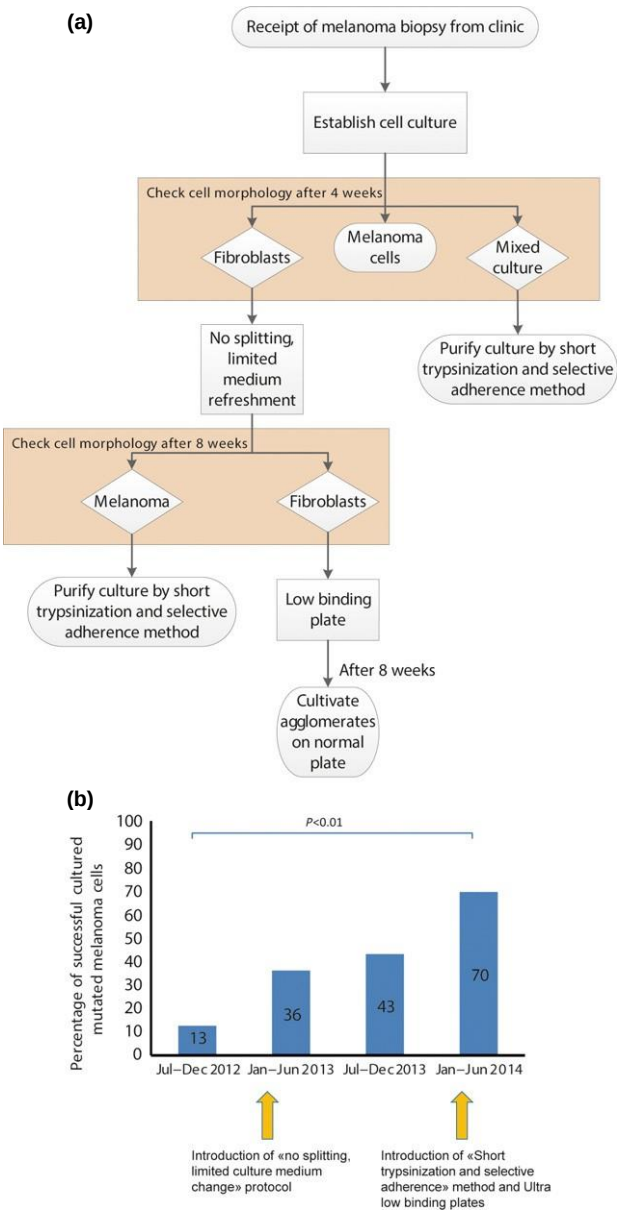

Figure 2. A new protocol for isolating melanoma cells from native material. (a) A conditional workflow based on cell morphology and oncogenic driver mutation status. It uses the principles of no splitting, limited medium refreshment, short trypsinization times and selective adherence to enrich for melanoma cells. (b) The amount of successful melanoma cell cultures in 2012, 2013 and 2014, when the new culture protocol was applied to the biobank collection, followed by Sanger sequencing of the patient's known BRAF or NRAS mutation status.

resulting in a recovery rate of 60% overall for the combined protocols.

Conditional treatment protocols increase the success rate of large-scale biobanking programmes

Since the beginning of 2014, we applied a new cell culture workflow (Fig. 2a) that included the different methods for retrieving melanoma cells out of fibroblast-rich cultures. This resulted in a significant improvement from 13% using the standard protocols to 70% with our optimized workflow in establishing melanoma cultures (Fig. 2b), compared with the previous year when the standard protocol was still used.

Discussion

Currently, most *in vitro* research is carried out on fast-growing, adherent and robust cell cultures or cell lines, because these are the easiest to isolate and maintain. This is readily seen in our spike experiment in which fast-growing melanoma cells rapidly overtake the fibroblasts in culture. However, the slow-growing melanoma cells that are much more difficult to retrieve are largely underrepresented by most biobanking efforts (14). However, these cells may have important consequences for tumor progression or treatment response. Here we present an optimized protocol for establishing heterogeneous melanoma cell cultures out of patient biopsies, which reduces the chance of fibroblast overgrowth. Our method improves the basic *in vitro* tools available to study melanoma biology by better representing the heterogeneous cell types present in melanoma tumors.

Our proposed workflow starts with a 4-week culture period using the standard protocol. It may not always be necessary to use special techniques to obtain slow-growing melanoma cells. For instance, from tissues with little fibroblast contamination such as in brain metastases, we successfully obtained slower growing melanoma cells with the standard protocol. However, for some samples, this standard protocol does not suffice, and then, the optimized methods could be used to retrieve the melanoma cells from these samples as well. Application of our combined methods to 20 cultures that had failed the standard culturing protocols resulted in the retrieval of 11 additional cultures using the 'no splitting' and 'selective adherence' methods and 1 culture using the 'low-binding plate' method, for a total success rate of 60%. While no method was able to retrieve mutated melanoma cells from 8 of the 20 cultures, it is possiblethat these early cultures never contained melanoma cells, orsome other method would be necessary to enrich for melanomas from these cultures.

By applying this optimized workflow to our live-cell biobanking programme, we improved our recovery rates from 13% to 70% in establishing melanoma cell cultures from native material. In addition to confirming the oncogenic mutation status or morphology of over 250 early passage melanoma cultures, we also have a biobank of more than 1000 different unconfirmed early passage melanoma cell cultures from consenting patients with diverse clinical features and treatment histories. As we have shown that it is possible to recover melanoma cells from impure cultures that may have failed quality control steps from standard protocols, our collection represents an immense resource for investigating how melanoma heterogeneity contributes to tumor progression and therapeutic resistance.

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Author contribution

The article was written by MIGR and MPL. MIGR, MM, TK, AL, AF and CS performed the experiments. DW was involved in analysing the data and formatting the manuscript. RD and MPL contributed essential reagents and supervised the project.

Conflict of interests

The authors have declared no conflicting interests.

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7. Paper 2: Co-existence of BRAF and NRAS driver mutations in the same melanoma cells results in heterogeneity of targeted therapy resistance

Research Paper

Co-existence of *BRAF* and *NRAS* driver mutations in the same melanoma cells results in heterogeneity of targeted therapy resistance

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ABSTRACT

Acquired chemotherapeutic resistance of cancer cells can result from a Darwinistic evolution process in which heterogeneity plays an important role. In order to understand the impact of genetic heterogeneity on acquired resistance and second line therapy selection in metastatic melanoma, we sequenced the exomes of 27 lesions which were collected from 3 metastatic melanoma patients treated with targeted or non-targeted inhibitors. Furthermore, we tested the impact of a second NRAS mutation in 7 BRAF inhibitor resistant early passage cell cultures on the selection of second line therapies.

We observed a rapid monophyletic evolution of melanoma subpopulations in response to targeted therapy that was not observed in non-targeted therapy. We observed the acquisition of *NRAS* mutations in the BRAF mutated patient treated with a BRAF inhibitor in 1 of 5 of his post-resistant samples. In an additional cohort of 5 *BRAF*-inhibitor treated patients we detected 7 NRAS mutations in 18 post-resistant samples. No NRAS mutations were detected in pre-resistant samples. By sequencing 65 single cell clones we prove that *NRAS* mutations co-occur with *BRAF* mutations in single cells. The double mutated cells revealed a heterogeneous response to *MEK, ERK, PI3K, AKT and multi RTK* - inhibitors.

We conclude that BRAF and NRAS co-mutations are not mutually exclusive. However, the sole finding of double mutated cells in a resistant tumor is not sufficient to determine follow-up therapy. In order to target the large pool of heterogeneous cells in a patient, we think combinational therapy targeting different pathways will be necessary.

INTRODUCTION

The MAPK pathway, consisting of *RAS-RAF-MEK-ERK*, is a highly conserved signaling cascade in eukaryotic cells conserved from yeast to humans with many vital cellular functions, such as proliferation, differentiation, migration, and apoptosis [1]. About one-third of cancers have a deregulated MAPK pathway, either due to overexpression of receptor tyrosine kinases (RTKs), increased production of activating ligands, activating mutations in RTKs, *RAS* or *RAF* or to failure of pathway control mechanisms [1]. In cutaneous melanoma, deregulation of the MAPK pathway is mainly caused by a hyperactive mutation in *BRAF* (50% of cases) or *NRAS* (15% of cases), highlighting the important role of controlled MAPK signaling for melanocyte homeostasis [1, 2].

Targeting a hyperactivated MAPK pathway driven by mutated *NRAS* or *BRAF* with specific *BRAF*- and *MEK* inhibitors, increases the median overall survival from metastasized melanoma patients from 9 months with no therapy to approximately 14 months with successful inhibitor treatment [3]. Unfortunately, resistance to MAPK inhibition almost invariably develops [4]. Several resistance mechanisms have been described so far, which can roughly be divided into those that reactivate the MAPK pathway by circumventing the inhibitory effects of the MAPK inhibitors, or ones that activate alternative signaling pathways [5].

In the case of *BRAF* inhibitors, Shi et al identified reactivation of the MAPK pathway (70% of cases), mostly in the form of additional NRAS or KRAS mutation (18% and 7% of cases, respectively), CDKN2A loss (7% of cases), mutant BRAF amplification (19% of cases) or BRAF alternative splicing (13% of cases) as the most common resistance mechanisms. They also identified the PI3K-PTEN-AKT pathway as the second important resistance pathway (22% of their post-treatment samples contained mutations in PI3K-AKT regulatory genes) [5].

One of the more prevalent mechanisms is an additional mutation in *NRAS*, leading to reactivation of the MAPK pathway [6]. However, it has been published that *NRAS* and BRAF mutations are mutually exclusive in single cells due to self-induced apoptosis by sustained hyper-activation of the MAPK pathway [7, 8]. Consequently, resistant tumors of patients that contain both mutations concurrently may be comprised of several mutually-exclusive subclones with either the activating *BRAF* or *NRAS* mutations [7]. A recent paper showed that both mutations can co-occur in a small area (of approximately 10,000 cells) selected by laser microdissection [9], although this does not prove that the mutations can co-occur within single cells. Likewise, although double-mutated *NRAS/BRAF* melanoma cultures have been previously reported, these may still represent heterogeneous mixtures of singly-mutated melanoma cells [10, 11, 12], or may have arisen artificially through *in vitro* drug treatment and selection experiments [13].

Within a patient, various small populations of tumor cells (i.e., subclones) evolve during disease progression, which exhibit different genotypes and/or phenotypes ([14, 15, 16]). Due to these different tumor subclones within a patient (intra-patient heterogeneity), it is believed that diverse resistance mechanisms can co-exist within one patient [17, 5]. However, where these resistance mechanisms originate from and how they evolve under treatment remains poorly understood [6, 11].

To better characterize the evolution of intra-patient heterogeneity under different treatment regimens, we performed exome sequencing on multiple samples from 3 stage IV melanoma patients (cohort 1) who each received a different therapy (BRAF inhibitor (patient 1), MEK inhibitor (patient 3) or multi-receptor tyrosine kinase (patient 2)) but progressed quickly under treatment. We used formal phylogenetic methods on tumor DNA to model the evolution of intra-patient heterogeneity from primary tumors to each individual metastasis for the targeted and non-targeted therapies. In addition, we could detect the presence of an *NRAS* mutated subclone in 1 of 5 treatment-resistant tumors from the BRAF inhibitor resistant patient (patient 1). Single cell clone sequencing from the cell culture generated from this treatment resistant tumor revealed the co-occurrence of a BRAF and NRAS mutation in a single cell. This was confirmed in an additional group of 5 patients (patient 4, 5, 6, 7 and 8, cohort 2) from whom tumors after BRAF inhibitor treatment were collected and where cell cultures were generated from these tumors and showed secondary NRAS mutations. Sequencing of 65 clonal populations derived from 4 of these cell cultures showed the presence of both activating MAPK mutations in all but one subclone. Further *in vitro* work with these double-mutated cell cultures demonstrated sensitivity to *BRAF* inhibition, but heterogeneous responses to downstream MAPK inhibition, as well as to PI3K pathway inhibitors and the multi-receptor kinase inhibitor Pazopanib.

RESULTS

Tumor-type dependent, intra-patient heterogeneity

We sequenced whole exomes of 27 samples from three metastatic melanoma patients (cohort 1) with different mutational statuses and different treatments (Table 1 and Figure 1A–1F). For all patients we performed exome sequencing on all of their samples and confirmed their mutational status (*BRAF*^{V600E} mutated, *BRAF*^{WT}/*NRAS*^{WT} or *NRAS*^{Q61K} mutated for patient 1, 2 and 3, respectively) (Table 1, Figure 1, Supplementary. Table S1). In addition to these driver mutations, we looked for other mutated onco- and tumorsuppressor genes: in patient 2 we identified a nonsynonymous germline mutation in the Melanocortin receptor *MC1R*^{V92M} and in patient 3 a nonsynonymous germline mutation in the Microphthalmia-associated transcription factor *MITF*^{E318K} (data not shown). By using EXCAVATOR and CONTRA algorithms we detected a high number of copy number variations (CNVs) in many chromosomes, with some samples exhibiting large losses throughout the genome (Figure 1G–1I).

Whole-exome phylogenetic analysis identifies monophyletic evolution of therapeutic resistance

In order to investigate the evolutionary relationship between tumor sites within individual patients, we used phylogenetic algorithms to group tumor samples based on their total single nucleotide variants (SNVs) and copy number variations (CNVs) (Figure 2, Supplementary

Table 1: Overview table of all the patients mentioned in this manuscript

	Patient	Gender	Date of birth	Mutation identified in clinic	Treatment	Histomaterial	Mutations	Cell line	Date of original biopsy from cell line	Mutations single cells	Other mutations Cell line (MelArray)
Cohort 1 Whole exome sequencing for investigating melanoma evolution patterns under targeted vs non-targeted therapy	Patient 1	male	11.01.1975	BRAF V600E	LGX818 (encorafenib) Aug 2012 - Dec 2012 Drug compliance questionable	Primary Early met 1- Early met 2- Early met 3- Late met 1- Late met 2- Late met 6- Late met 4- Late met 5- Neavus 1 Neavus 2	Sanger Sequencing: NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS Q61K, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E whole exome sequencing, results see Figure 1, suppl. Table 1, Figure 2, suppl. Table 2, suppl. Table 3	MM121224	1 month after therapy skin metastasis	BRAFV600E NRAS Q61K 23 from 23 clones	ASPM C2826F GNAQ M59L HER/C2 D15N KDR Y1057er KMT2A S2819F KMT2C Q755Ter ROS1 E1291K SMARCA4 R978Q TERT promoter-146C>T
	Patient 2	male		BRAF WT NRAS WT	Pazopanib Oct 2012-Dec 2012	Primary (3 sites) Late met 1 Late met 2 Late met 3 Late met 4 Late met 5 Late met 6	whole exome sequencing, results see Figure 1, suppl. Table 1, Figure 2, suppl. Table 2, suppl. Table 3	-	-	-	-
	Patient 3	male		NRAS Q61K	MEK162 (binimetinib) Jun 2013 - Mar 2013	Primary (2 sites) Early met 1 Late met 1 Late met 2 Late met 3	whole exome sequencing, results see Figure 1, suppl. Table 1, Figure 2, suppl. Table 2, suppl. Table 3	-	-	-	-
Cohort 2 Validating occurence of BRAF/NRAS double mutated cells	Patient 4	male	28.01.1959	BRAF V600E	LGX818 (encorafenib) Jul 2014 - Feb 2015	Primary Late met 1 Late met 2 Late met 3 Late met 4	NRAS WT, BRAF V600E NRAS Q61R, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS Q61R, BRAF V600E	MM150423	2 months after therapy liver metastasis	BRAFV600E NRAS Q61R	ALK D885A DNMT1 L828P PLCE1 E88K
	Patient 5	male	16.12.1940	BRAF V600K	PLX4032 (vemurafenib) Oct 2012 - Mar 2014 LGX818 (encorafenib) in combination with MEK162 (binimetinib) Jul 2014 - Feb 2015	Late met 1-	NRAS G12A, BRAF V600K	MM140307	1 month after BRAF inhibitor monotherapy 4 months before combination therapy skin metastasis	BRAFV600K NRAS G12A 16 from 16 clones	AKT3 R249H ASPM P223H CDC42 P87S CYP7B1 R370C EIF1AX G15D FGFR4 E560_R565del KMT2C E864G KMT2C G824D KMT2C K399N MAP2K2 F57L MAP3K1 D1084L_fs_Ter13 MAP3K9 S334C NTRK1 D537N PIK3R1 S400F PTPRK Q1364Ter ROS1 E1642K TRRAPA2383V TUSC3 Q169Ter
	Patient 6	female	02.03.1951	BRAF V600E	PLX4032 (vemurafenib) Aug 2013 - Aug 2014	Primary Late met 1 Late met 2	NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS Q61K, BRAF V600E	MM140906	1 month after therapy brain metastasis	BRAFV600E NRAS Q61R	N/A
	Patient 7	male	26.10.1956	BRAF V600E	MEK162 (binimetinib) Nov 2011 - Feb 2012 PLX4032 (vemurafenib) Feb 2012 - Jun 2013	Early met 1 Early met 2 Early met 3	NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E	M130903	8 months after PLX4032 subcutaneous metastasis	BRAFV600E NRAS Q61H 18 from 18 clones	CDKN2A R80Ter KMT2C Y987H KMT2D S3691F
	Patient 8	female	14.09.1974	BRAF V600E	LGX818 (encorafenib) in combination with MEK162 (binimetinib) Jul2015 - Aug 2015	Early met 1 Early met 2 Early met 3 Early met 4 Early met 5 Early met 6 Late met 1 Late met 2 Late met 3 Late met 4 Late met 5 Late met 6 Late met 7 Late met 8 Late met 9 Late met 10 Late met 11	NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS Q61R, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS Q61R, BRAF V600E NRAS WT, BRAF V600E NRAS Q61R, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E NRAS WT, BRAF V600E	MM150849	1 month after therapy skin skull	BRAFV600E NRAS Q61R 7 from 8 clones	CDKN2A D146M_fs_Ter9 KMT2D P1170L PLCB1 E60K PTPRJ R306G SPRY4 G93R TP53 P151A TSC2 S556F
								MM150850	1 month after therapy ovary	BRAFV600E NRAS Q61K	CDC42 V36F CDKN2A D146M_fs_Ter9 KMT2C K3870R KMT2C G892R KMT2C T820I KMT2D P1170L MAP3K5 T989D_fs_Ter35 PIKFYVE Q663R_fs_Ter33 PLCB1 E60K ROS1 K832R_fs_Ter2 SPRY4 G93R TP53 P151A TSC2 S556F

Cohort 1 consists of three patients on whose immunohistochemistry material we performed whole exome sequencing in order to obtain insight in the evolution of melanoma progression under targeted and non-targeted therapy. The finding of a double mutated BRAF and NRAS cell culture was validated in cell cultures from cohort 2. Cohort 2 consists of 5 patients whose cell cultures showed mutations in both NRAS and BRAF. Sanger sequencing of all of the immunohistochemistry blocks from these patients could identify the double mutation only in the post-treatment samples.

Table S2). As opposed to the phylogenetic tree of patient 2 biopsies, the evolutionary trees from patient 1 and 3 showed monophyletic clades for the post-resistance samples (i.e. late metastases), meaning that these originated from only one node (Figure 2). However, no known and shared mechanism of resistance to *BRAF*-inhibitor or *MEK*-inhibitor treatment could be identified (Supplementary Table S2). Confidence is shown by bootstrap supports (arrow) which reflects the percentage of bootstrap trees also placing the clade at the endpoints of that branch. The tree of patient 2 (*BRAF*^{WT} and *NRAS*^{WT}, patient received non-targeted therapy) did not show this strong monophyletic clade of late tumor metastases, instead the post-resistant samples originated from multiple nodes (Figure 2, arrows). Since there might be multiple different resistance mechanisms present in one patient, we also sought to identify explanatory protein-coding changes in any of the post-treatment samples. However, no known mechanisms of resistance were identified in the exome data of any tumor in the three patients except for the *NRAS*^{Q61K} mutation that was present in a cell line (MM121224) derived from a resistant metastasis from patient 1 (Figure 2).

Resistant subclones are present heterogeneously and at low frequencies

In order to determine the origin of this *NRAS*^{Q61K} mutation from patient 1 we performed digital PCR as well as ultra-deep sequencing on all tumor samples (data not shown, and Figure 2D). Only one cell culture (MM121224) and one biopsy where the cell culture was derived from had an activating mutation in exon 2 of the *NRAS* gene (*NRAS*^{Q61K}), with a range from 5,473× coverage of the *NRAS* exon to 26,416× coverage by next generation sequencing (Figure 2D, and Supplementary Table S3). No other sample from this patient had this activating mutation, suggesting other resistance mechanisms to be involved in the metastases of the same patient. Using the program deepSNV, a diverse series of other *NRAS*

coding mutations in exon 2 was present significantly in all samples at low subclonal frequencies compared to the germline blood reference from the same patient (Figure 2D and 2E, Supplementary Table S3). Interestingly, the allele carrying the *NRAS*^{Q61K} mutation was only present at a frequency of about 6% in early passage cultures of the MM121224 cell line from this patient (Figure 2D–2F).

Two activating MAPK mutations are present in single melanoma cells

To determine the frequency of double mutations in early passage cultures in general in a larger patient cohort, we Sanger sequenced the *NRAS* locus in all *BRAF*-mutated cell cultures generated from 2013—2015 archived in our melanoma biobank [18]. In this way, we identified an additional 8 double-mutated cell cultures, derived from 7 different patients, bringing the total to 9 out of 122 cell cultures (7.4%). Of these 9 cell cultures, 7 had an activating *BRAF* mutation at position 600 and a co-occurring activating *NRAS* mutation at position 61 or 12 (Table 1). Two of these cell cultures had a *BRAF* mutation at another position, namely one culture contained the double mutation *BRAF*^{D594H}/*NRAS*^{Q61R} and one culture had the double mutation *BRAF*^{L597R}/*NRAS*^{Q61R} (Data not shown). The *BRAF*^{D594H} is an inactivating mutation, and a double mutation of this sort is already described elsewhere [19]. The *BRAF*^{L597R} is a less prevalent activating mutation for which less information is available. We therefore decided to focus on the six patients (patients in cohort 2, and patient 1 from cohort 1, see Table 1) with cell cultures that had a *BRAF* mutation at position 600 and a co-occurring *NRAS* mutation at position 61 or 12.

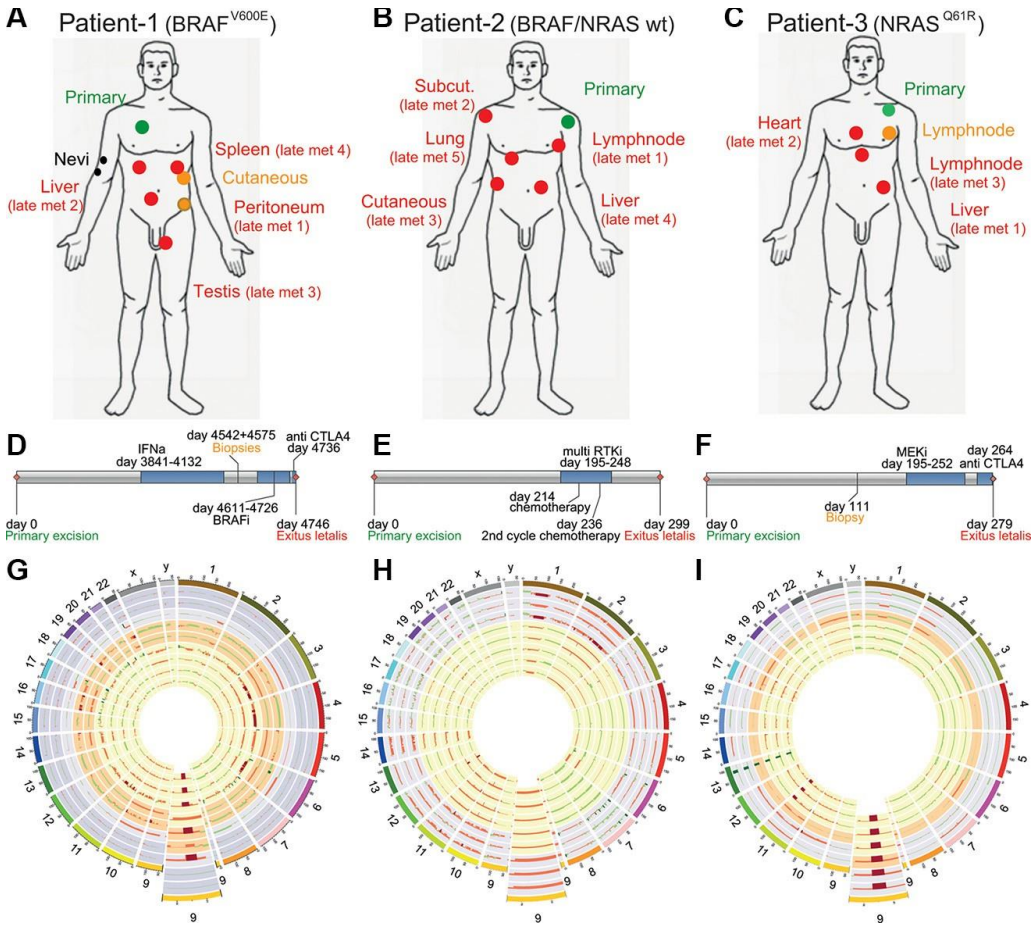


Figure 1: Patient cohort (A, D) Patient 1 had a *BRAFV*^{600E} mutated melanoma, samples were collected pre- and post LGX818 (encorafenib) treatment and included the primary tumor (green), two dysplastic nevi (black), two early metastases (orange) and 4 late metastases after tumor relapse (red). (**B, E**) Patient 2 had a melanoma WT for *BRAF* and *NRAS*. Samples were collected pre- and post non targeted multi RTK inhibitor (pazopanib), and included the primary tumor (green) and five late metastases (red). (**C, F**) Patient 3 had a *NRAS*^{Q61R} mutated melanoma, samples were collected pre-and post MEK162 (binimetinib) treatment and included the primary tumor (green), one early metastasis (orange) and three late metastases (red). (**G**, **H**, **K**) Every ring shows the CNVs detected of one biopsy, The enlarged regions show a commonly lost region in chromosome 9 which is coding for the tumor suppressor *CDKN2A*. (G) Patient 1, rings from outside to the center represent two nevi in the two outermost circles followed by the primary tumor, the two early metastases and finally the late metastases 1 to 4. (H) Patient 2, rings from outside to the center represent primary tumor samples 1 to 3 and the late metastases 1 to 5. (**I**) Patient 3, rings from outsided to the center represent the primary tumor samples 1 and 2, one early metastases and the late metastases 1 to 3.

We asked if the double-mutated cell cultures consisted of two exclusive populations of cells (one with *BRAF* and another with *NRAS* mutations), or if both mutations were present in single melanoma cells. To distinguish between these possibilities, we generated single-cell clones of 4 double mutated cell cultures (from patients 1, 5, 7 and 8) and confirmed by Sanger sequencing the presence of both $BRAF^{V600E/K}$ and $NRAS^{Q61K/H/R \text{ or } G12A}$ mutations in 65 independently derived colonies (Table 1).

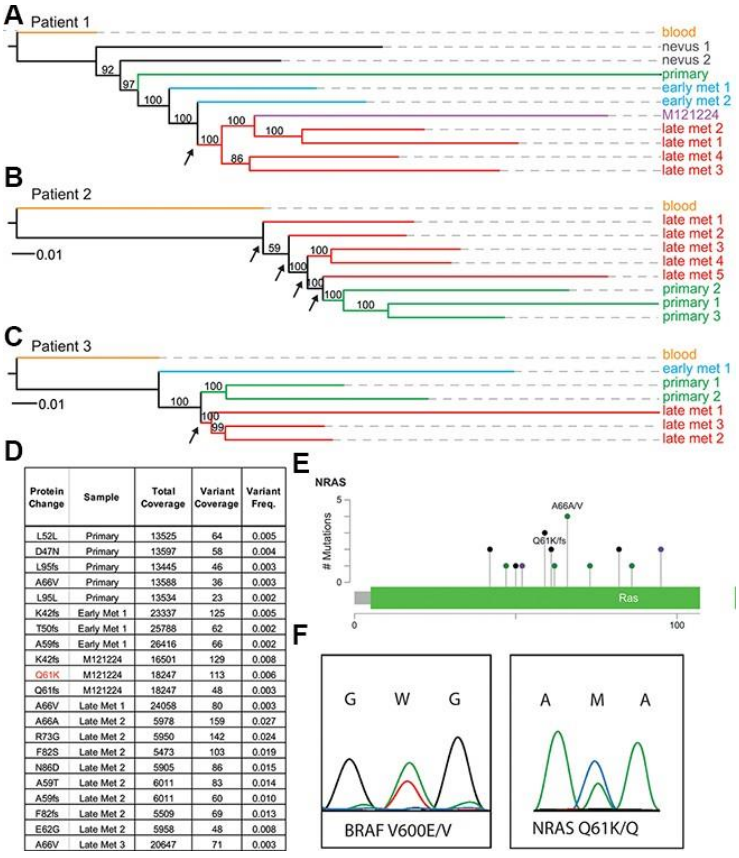
Both alleles (mutated and WT) from *BRAF* and *NRAS* were found to be expressed with Sanger sequencing of cDNA and RNA-seq (data not shown).

Double mutations occur heterogeneously within patients after targeted therapy

In order to investigate the evolution of the *NRAS/BRAF* double mutated cancer cells within a patient, we analyzed tumor DNA for the presence of double mutations in all available histological and frozen material from patients 4, 5, 6, 7 and 8 before and after *BRAF* inhibitor treatment (Table 1). For patients 4, 5 and 6 we could confirm the presence of the additional *NRAS* mutation in the post-treatment samples from which the cell culture was derived. In patient 8, we had generated cell cultures from Late met 1 and Late met 2. In Late met 1 we could detect the $BRAF^{V600E}$ $NRAS^{Q61R}$ double mutation, however in Late met 2 we could not detect the additional $NRAS^{Q61K}$ mutation. For patient 7, the histology block corresponding to the tumor from which the cell culture was derived was no longer available. In patient 4 and patient 8, we could also detect the *NRAS/BRAF* double mutation in additional post-treatment metastasese. Interestingly, the *NRAS* mutation could not be detected in any of the pre-treatment biopsy samples.

Double-mutant cells have heterogeneous MAPK pathway inhibitor and alternative pathway responses

In order to gain more insight into the biology of *NRAS/BRAF* double mutated cells, we performed viability assays with (control) single and double mutated cell cultures under MAPK inhibitor treatment. Double-mutated cell cultures from all 6 identified patients were resistant to three different *BRAF* inhibitors (Figure 3A, Table 2). The response to *MEK*



Protein Change	Sample	Total Coverage	Variant Coverage	Variant Freq.
L52L	Primary	13525	64	0.005
D47N	Primary	13597	58	0.004
L95fs	Primary	13445	46	0.003
A66V	Primary	13588	36	0.003
L95L	Primary	13534	23	0.002
K42fs	Early Met 1	23337	125	0.005
T50fs	Early Met 1	25788	62	0.002
A59fs	Early Met 1	26416	66	0.002
K42fs	M121224	16501	129	0.008
Q61K	M121224	18247	113	0.006
Q61fs	M121224	18247	48	0.003
A66V	Late Met 1	24058	80	0.003
A66A	Late Met 2	5978	159	0.027
R73G	Late Met 2	5950	142	0.024
F82S	Late Met 2	5473	103	0.019
N86D	Late Met 2	5905	86	0.015
A59T	Late Met 2	6011	83	0.014
A59fs	Late Met 2	6011	60	0.010
F82fs	Late Met 2	5509	69	0.013
E62G	Late Met 2	5958	48	0.008
A66V	Late Met 3	20647	71	0.003

Figure 2: Whole-exome phylogenetic trees of patient biopsies. Branch-lengths represent relative distances based on SNVs and indels, and the branches are colored according to biopsy type. Maximum likelihood phylogenetic trees are rooted by the blood sample for patient 1 (**A**), patient 2 (**B**), and patient 3 (**C**). Node supports are given as bootstra *p* values, with greater than 50% considered to be strong support. (**D**, **E**) deep sequencing results of the *NRAS* exon 2 locus in multiple samples from patient 1. (**F**) the double $BRAF^{V600E}$ and $NRAS^{Q61K}$ mutation is present in colonies derived from single cells.

Table 2: Overview table of the responses of the different double mutated cell cultures on various therapies

	MM121224		MM150423		MM140307		MM140906		M130903		MM150849		MM150850	
Drug	IC50 monotherapy	Synergism	IC50 monotherapy	Synergism	IC50 monotherapy	Synergism	IC50 monotherapy	Synergism	IC50 monotherapy	Synergism	IC50 monotherapy	Synergism	IC50 monotherapy	Synergism
PLX4032 (BRAF inhibitor)	8 uM		> 10 uM		> 10 uM		> 10 uM		> 10 uM		> 10 uM		> 10 uM	
LGX818 (BRAF inhibitor)	>200 nM		> 200 nM		> 200 nM		> 200 nM		> 200 nM		> 200 nM		> 200 nM	
GSK2118436 (BRAF inhibitor)	> 200 nM		> 200 nM		> 200 nM		> 200 nM		> 200 nM		> 200 nM		> 200 nM	
MEK162 (MEK inhibitor)	0.1 uM		1 uM		> 1 uM		> 1 uM		0.5 uM		0.5 uM		> 1 uM	
SCH772954 (ERK inhibitor)	0.05 uM		1 uM		0.5 uM		> 1 uM		0.2 uM		0.5 uM		> 1 uM	
GDC0941 (PI3K inhibitor)	500 nM		1.5 uM		500 nM		1 uM		500 nM		> 5 uM		N/A	
GSK690693 AKT inhibitor)	2 uM		100 nM		> 2 uM		> 2 uM		> 2 uM		> 2 uM		N/A	
Rapamycin (mTOR inhibitor)	0.05 nM		> 1 nM		> 1 nM		> 1 nM		0.8 nM		> 1 nM		N/A	
Pazopanib (multi RTK inhibitor)	> 2 uM		> 2 uM		> 2 uM		> 2 uM		> 2 uM		> 2 uM		N/A	
BRAFi + MEKi		+		-		-		-		+		-		N/A
ERKi + MEKi		-		+		+		++		+		-		N/A
MEKi + PI3Ki		+++		++		+		++		++		+++		N/A
MEKi + AKTi		++		++		+++		+++		++		+++		N/A
MEK + Rapamycin		+		+		+++		+++		+		++		N/A
MEK + Pazopanib		+		-		++		-		++		-		N/A

A + indicates synergism of combination treatment, with ++ and +++ being a stronger effect. A – indicates no synergy.

and *ERK* inhibitors was heterogeneous. The cell cultures MM140906 and MM150850 were resistant to MEK and ERK inhibitors, whereas other cell cultures were sensitive or partially resistant for one or for both of the inhibitors (Figure 3A, Table 2). We also tested the response for different inhibitors of the PI3K-AKT pathway, as this pathway is often involved in MAPK pathway inhibitor resistance [5], and for the multi-RTK inhibitor Pazopanib. Here the cells also showed a heterogeneous response, except for the multi-RTK inhibitor, for which all cell cultures were resistant (Figure 3A, Table 2). Therapies combining a MEK inhibitor with different PI3K pathway inhibitors worked synergistically in all cell cultures, albeit with different strengths (Table 2). As it was previously thought that *BRAF* and *NRAS* mutations are mutually exclusive due to the growth disadvantage of double mutated cells [8], we analyzed the proliferation rate of double-mutated cells *in vitro*, compared to single mutated control cell cultures (Figure 3B). Although the cell cultures MM121224 and MM140307 showed a higher proliferation rate compared to the control cell cultures, the other double-mutated cells had reduced proliferation rates.

As a downstream read-out for *BRAF* and/or *MEK* activation, western blot analysis for total *ERK* and phosphorylated *ERK* was performed in the presence of *BRAF*, *MEK*, or *ERK* inhibition. This showed that basal *pERK* levels were mostly higher in the double mutated cells compared to the single *NRAS* mutated control cell cultures (but not in MM150423) (Figure 3C). Upon treatment with the *BRAF* inhibitor LGX818 (encorafenib), the *pERK* levels of MM121224, MM150423, MM150849 and MM140906 stayed the same compared to the untreated control, whereas the levels of MM140307, MM150850 and M130903 decreased (Figure 3C). Levels of *pERK* expression upon *MEK162 (binimetinib)* treatment decreased in all cell cultures except for MM140906. Upon *ERK* inhibition, *pERK* levels were reduced in all cell cultures.

When we compared pERK levels and pAKT levels between the different double mutated cell cultures without treatment, we found that MM140307, MM140906, MM150849 and MM150850 expressed relatively high levels of pERK, whereas M130903, MM150423 and MM121224 express relatively low levels (Figure 3D). pAKT expression is relatively constant between the different cell cultures, except MM150849, which has a relatively high expression (Figure 3D)

DISCUSSION

Genetic or transcriptional heterogeneity in tumors is a major obstacle to obtaining durable responses to

targeted therapy for metastatic melanoma. In order to better understand how individual cancer patients respond to standard therapies, we conducted multiple-sample, whole-exome sequencing from multiple time-points in 3 patients receiving different therapeutic regimens.

The sequencing results were used to infer the evolutionary relationships between the tumors within each patient, and to determine how each therapeutic regimen affected the evolution of genetic heterogeneity. Unlike previous studies that showed a branching evolution of clones subsequent to targeted therapy, we could see a strong, well-supported monophyletic evolution of metastases following both *BRAF* and *MEK* inhibitor treatment and relapse with phylogenetic analysis [5]. In contrast, patient 2, who received a multi-kinase inhibitor (i.e. pazopanib), did not have a monophyletic topology of late tumor metastases, which is suggestive of genetic drift between the late metastases. Despite the monophyletic segregation of late metastases in the patient who received the *BRAF* inhibitor, no known genetic mechanism of resistance was shared between all sequenced biopsies accounting for the inter-patient heterogeneity and subsequent treatment difficulties. In fact, the additional activating mutation in $NRAS^{Q61K}$ found in patient 1 with a *BRAF* mutation background was only present in a single metastasis of patient 1 and absent in all other resistant tumor samples from that patient. This is consistent with previously published data showing heterogeneity in resistance mechanisms within individual patients [5].

We went on to check if the double mutation could also be found in the cell culture obtained from this resistant metastasis, and if these mutations occurred in the same cell. By isolating and sequencing colonies derived from 23 single-cell clones of the resistant late metastasis 6 from patient 1, we could show for the first time that both activating MAPK mutations (NRAS and BRAF) were present in a single tumor cell.

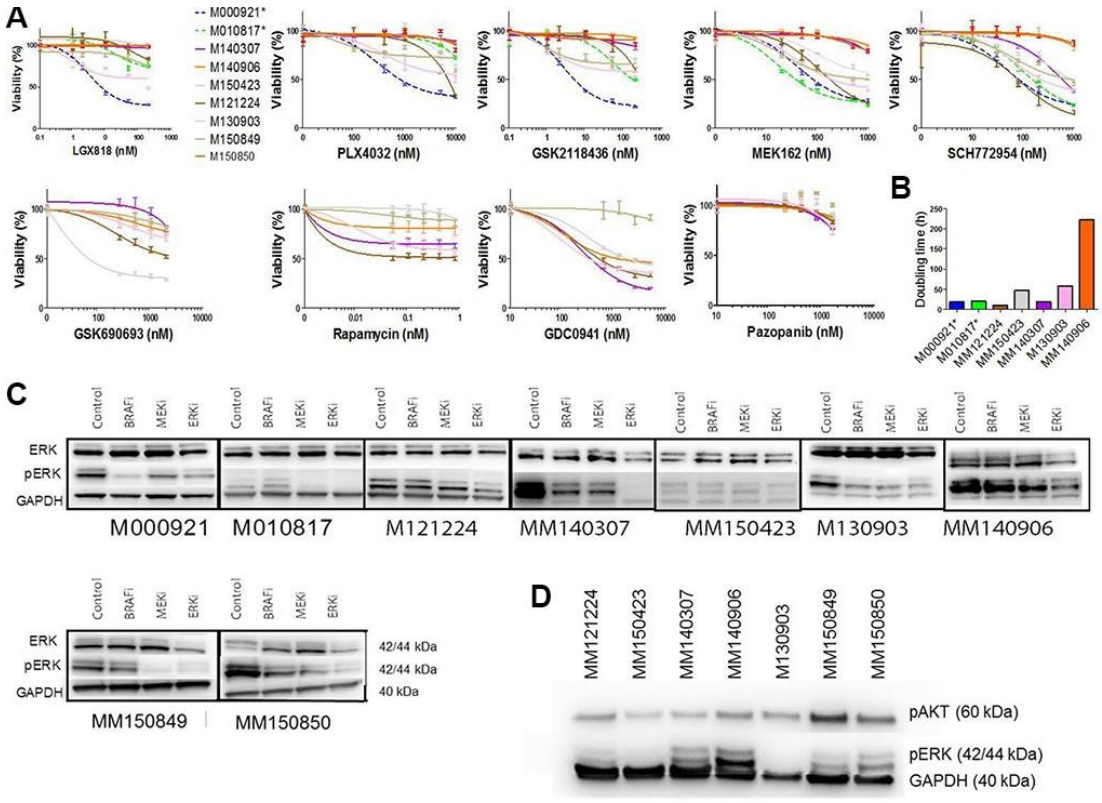


Figure 3: Viability and proliferation assays of double mutated cells. (**A**) Viability assays of double mutated cells for different *MAPK* inhibitors and inhibitors from the PI3K-AKT pathway as well as a *multi-RTK* inhibitor. Single mutated control cell cultures are M000921 ($BRAF^{V600E}$) and M010817 ($NRAS^{Q61R}$), indicated in dotted lines. The double mutated cell cultures are indicated in solid lines. MM121224 ($BRAF^{V600E}$, $NRAS^{Q61K}$) derives from patient 1, MM140307 ($BRAF^{V600K}$, NRASG12A) derives from patient 5, MM140906 ($BRAF^{V600E}$, $NRAS^{Q61R}$) derives from patient 6, MM150423 ($BRAF^{V600E}$, $NRAS^{Q61R}$) derives from patient 4, M130903 ($BRAF^{V600E}$, $NRAS^{Q61H}$) derives from patient 7, MM150849 ($BRAF^{V600E}$, $NRAS^{Q61R}$) and MM150850 ($BRAFV^{600E,}$ $NRAS^{Q61K}$) are both derived from patient 8. (**B**) Doubling time of double mutated cells under standard culturing conditions. Single mutated control cell cultures are indicated with stars. (**C**) Western blots showing *pERK* and *ERK* levels under MAPK inhibitor treatment in double mutated cell cultures. Single mutated control cell cultures are indicated with stars. (**D**) Westernblot showing pERK and pAKT levels under basic conditions (no treatment) in the different double mutated cells.

In order to confirm our finding, we identified a second cohort of BRAF inhibitor resistant patients from whom we had obtained a double mutated cell culture. In screening 121 cells from our live-cell biobank, we could identify an additional 8 cell cultures with double *BRAF/NRAS* activating mutations, bringing the total to 9 out of 122 cell cultures. By sequencing colonies derived from single-cell clones, we confirmed the presence of the double mutation in 64 out of 65 colonies. However, in our standard biobank protocol we establish cell cultures without additional treatment, also when they are derived from a therapy resistant patient. Therefore, due to selection, this could be an underrepresentation of the actual number of double mutated subclones in a typical MAPK-inhibitor resistant tumor. Sequencing of colonies derived from single cell clones of three of these double-mutated cultures confirmed that all except for one colony contained both *BRAF* and *NRAS* mutations, thus confirming the presence of both activation mutations in the same cells. Sequencing of the additional immunohistochemistry blocks from these patients only identified double mutations in the post-treatment samples, confirming the finding in patient 1 from cohort 1.

To understand the general resistance mechanisms of the double-mutated cells, we conducted viability assays with different MAPK inhibitors. The double-mutated cells grew in normal culturing conditions, notably without any MAPK inhibition, were all resistant to *BRAF*-inhibitors, but showed heterogeneity in their response to *MEK* or *ERK* inhibition, possibly because of co-existing mutations in other pathways. Combination treatment with MEK and BRAF inhibitors, as it is now clinical practice, showed synergism in MM121224 and M130903, but no synergistic or additional effect in the other cell cultures,Suggesting that simultaneous or second-line treatment with other MAPK-pathway inhibitors might still be effective in controlling progression in selected patients, but not in all. However, a MEK inhibitor combined with a PI3K, AKT or mTOR inhibitor was synergistic in all of the cell cultures, albeit with different strength. It has to be kept in mind however, as the double-mutated genotype was only present in one or two metastases from each patient, it is likely not the most important resistance mechanism in these patients and the efficacy of these second-line or combination treatments in controlling overall tumor burden is questionable. Since no common mechanism of resistance was found in any patient, it is possible that the other resistant tumors activated different pathways.

Except for 1 cell line (MM150423), all double mutated cell lines showed higher expression of *pERK* at the basal level compared to the single *NRAS* mutated cell line M010817. However, the basal level of *pERK* among the double mutated cells varied, with relatively high expression in MM140307 and MM140906 and relatively low expression in MM150423. It has been argued that *BRAF* and *NRAS* mutations are mutually exclusive due to a growth deficit of double mutated cells, because of senescence-inducing high levels of *pERK* [8]. In our experiment, MM121224 and MM140307 grew faster than the single mutated control cell lines and MM140906, MM150423 and M130903 grew slower, not supporting the view the cells with high *pERK* level grow slower. However, *in vitro* growth behavior might not represent *in vivo* growth, for instance depending on how well the cells have adapted to a 2D culture system, what growth factors are present or missing in the cell culture medium compared to the *in vivo* situation and how well the immune system can control metastasis formation.

The *pERK* levels in the cell lines under treatment of various MAPK pathway inhibitors showed some discrepancy with the proliferation assay. Most profound was the strong reduction in *pERK* upon treatment with the *BRAF* inhibitor and *MEK* inhibitor in MM140307, although the cell line was resistant to these two inhibitors. We hypothesize that this is due to the very high *pERK* levels in this cell line at baseline, in such a way that even a strong reduction compared to baseline does not suffice to block the pathway. This would also be true for MM140906 under *ERK* inhibitor treatment, although the cell line is resistant to *ERK* inhibition, levels of *pERK* show a decrease compaired to the baseline, but the levels are still high. MM150423 *pERK* levels are relatively low in *MEK* and *ERK* treated cells, although the cell line is partially resistant to these inhibitors. However, the *pERK* levels in the *MEK* and *ERK* treated cells do not differ considerably from the untreated cells.

In this study, we show that known-resistance mechanisms are present at low frequencies and heterogeneously within individual patients. Furthermore, we show that finding an additional *NRAS* mutation in a tumor sample following *BRAF* inhibitor treatment could indicate the presence of a double mutated subpopulation that is not necessarily sensitive to *MEK* inhibition or *ERK* inhibition, rendering the *MEK* inhibitor therapy in all such cases suboptimal. This study indicates that genetic analysis of one tumor biopsy does not fully define the resistance mechanism for the whole patient, which has important implications for secondary therapy strategies in case of primary resistance.

MATERIALS AND METHODS

Patients and sample preparation

Patients were selected after written consent from the patient, given through the university biobank program according to ethical approval numbers 647 and 800. We collected surplus material before and after therapy at autopsy. Samples were processed immediately after collection to ensure best possible DNA and RNA quality. Primary cell cultures were established as described in [18].

Notably, upon generation of cell cultures, all cultures were kept under standard conditions without additional treatment, even if they were derived from a therapy resistant patient.

DNA was isolated from paraffin embedded tissue, fresh frozen tissue, cultured cells and PMCs stored in the biobank of the institute of Dermatology of the University Hospital of Zürich. Germline DNA from PBMCs was sequenced for all patients if available as a reference [20].

DNA from paraffin blocks was isolated using the FFPE DNA isolation kit from Qiagen (QIAamp DNA FFPE Tissue Kit #56404) and optimized protocols developed by Ultan McDermott at the Sanger institute. Prior to DNA isolation, each block was evaluated by a trained dermato-histopathologist, and punches were made in tumor regions to ensure reduced contamination with stromal tissue.

For DNA isolation from non-paraffin embedded samples we followed standard DNA isolation protocols published earlier.

Library preparation and sequencing

DNA quality was measured by an Agilent 2100 Bioanalyzer or Agilent 2200 Tapestation. One to three ug of high quality DNA was used to prepare the whole exome library using the Agilent SureSelect V4 or V5 kit. Sequencing was performed on an Illumina Hiseq 2000 machine in the Functional Genomics Center at University of Zürich. For the whole exome sequencing we sequenced 0.25 lanes per sample, paired-end, with 100 bp reads.

Whole exome sequencing analysis

Bioinformatics analysis was conducted with a modified GATK pipeline [21–23]. Quality control was done with „FASTQC" [24]. Alignment of the FASTQ file to the reference genome "hg19" [25] Lander, Linton et al. 2001) and transformation from SAM to BAM was done with "BWA" [26]. PCR duplicates were marked by MarkDuplicates from "Picard" [22], Local realignment around indels with RealignerTargetCreator (GATK), realigning with IndelRealigner (GATK), fix mate information with FixMateInformation (Picard), base quality score recalibration with Baserecalibrator (GATK) and PrintReads (GATK). Variant calling was done with UnifiedGenotyper (GATK). For annotation of the VCF files we used Annovar [27], Samtools [28] and Bedtools [29]. For data interpretation we used Microsoft Access, Microsoft Excel, Venny [30], ConSet [31] and IGV [32, 33].

For copy number analysis we used Excavator [34] and Contra [35], results were visualized with Circos [36].

SNVs were filtered according to the following read count criteria: A base must have at least four mutant reads and at least 10 total reads, if less than 10 total reads, at least half of them must be mutated. Also all SNVs with a phred-scaled quality score of < 50 were excluded from further analysis. A SNV was called somatic if the unfiltered blood sample from the same patient did not show any mutant read for this position.

Mutant allele ratios (MAR) were calculated by dividing mutant read counts by total read counts for each called SNV. Frequencies for these ratios were calculated and trendlines were plotted in Excel with the Moving Average method (period: 3). To reduce the number of false positive SNVs we applied more strict filtering on the private SNVs. Quality threshold was raised to a phred score of 100, and the SNV needed to have at least 10 total reads. Genes that had more than 8 SNVs were excluded.

Deep sequencing of PCR amplicons containing NRAS exon 2

DNA of 7 tumor samples (EMG P5 cell culture, M121224, 401/II, 404/II, 403, H12.684, H12.12640/1/B) were amplified with primers containing a NRAS specific sequence (see chapter sanger sequencing), adaptor sequences and a unique multiplex-identifier (MID) sequence (according to eurofins protocol). Each tumor sample analyzed is carrying therefore the adaptor sequence and a unique MID sequence. The PCR product was gel purified and 200 ng of each amplicon was sending for deep sequencing. Amplicons were subjected to Roche 454 sequencing using emulsions-PCR. Data were analyzed using DeepSNV [37].

Sanger sequencing

After DNA amplification of *NRAS* and *BRAF* with the following primers: *BRAF* forward: 5′CTAAGAGGAAAGATG AAGTACTATG reverse: 5′CTAGTAACTCAGCAGCATCTCAG *NRAS* forward: 5′GATAGGCAGAAATGGGCTTGA reverse: 5′ATCAT CCTTTCAGAGAAAATAATGC using a touchdown program going from 60 C to 55 C in 10 cycles, followed by 40 cycles at 55 C, the PCR product was diluted 100× and send to Microsynth for sequencing.

Generation of single cell clones and single cell clone sequencing

Cells were distributed over 96-well plate, containing 1 cell per well, via FACS cell sorting or serial dilutions. Cells were grown for several weeks under standard conditions [18] until visible colonies had formed. Then, medium was removed and wells were washed with PBS. Colonies were directly lysed in the well, with 10 ul lysis buffer (2.5% 1 M Tris pH 8.0 (Ambion), 0.1% 1.5 M EDTA (Sigma-Alderich), 0.25% Tween 20 (Sigma-Alderich), 1% proteinase K (Qiagen), Aqua dest.), and incubated at 55°C for 1 hour, followed by 5 min at 95°C. Afterwards, 10 ul 25 mM MgCl2 was added and the total volume was devided over 2 PCR reactions for NRAS and BRAF Sanger sequencing.

Cell sorting

Around 1×10^7 melanoma cells were resuspended in 100 µl FACS buffer (1% FBS, 5 mM EDTA pH 8, 0.01% NaN_3/ddH_2O in PBS). Cells were incubated for 20 minutes at 4°C with the following photosensitive antibodies: Anti-human MCSP-FITC (1:20 dilution) (Miltenyi Biotec 130-098-794, Bergisch Gladbach Germany) and Anti-human Fibroblasts/Epithelial-PE (1:200 dilution) (ABIN319868, Aachen Germany). After washing, cells were resuspended in 200 µl FACS buffer and sorted using the Aria IIb (BD Biosciences, Franklin Lakes, New Jersey, USA).

Phylogenetic analysis

Maximum Parsimony, Bayesian and Maximum likelihood (ML) phylogenies were constructed with the POSIX-threads version of RAxML v8.0.19 (7). To correct for among-site rate heterogeneity using the Γ distribution, we used an ascertainment bias correction and a general time reversible (GTR) substitution model. Four rate categories (ASC_GTRGAMMA model) were used to calculate the optimal tree. Node support was evaluated with 100 nonparametric bootstrap pseudoreplicates, they therefore indicate the percentage of bootstrap trees that contained a given internode branch.

Variants diagnostic for a given clade are defined as existing solely in that clade and nowhere else for that position. All leaves emanating from the node in question must share a variant and all other leaves must contain a different character for a variant to be diagnostic. Diagnostic variants can therefore also be termed an apomorphy.

Cell viability assay

1×10^4 cells were seeded and treated for 72 hours with different concentrations of either a BRAF inhibitor (PLX4032, LGX818 or GSK2118436), a MEK inhibitor (MEK162), an ERK inhibitor (SCH772984). DMSO treatment was used as a control. After 72 hours, the medium was removed and fresh RPMI1640 supplemented with 10% FCS and 8% MTT reagent (Sigma, 5 mg/ml in PBS) was added, and the cells were incubated at 37°C. After 1 hour, the RPMI1640 with MTT reagent was removed and 10% SDS (Sigma) and 95% isopropanol/ 5% Formic Acid (Sigma) (ratio 1:1) were added. After 5 min of incubation at 37°C, absorbance was measured at 595 nm (reference 620 nm) using a microplate reader.

Synergy calculations

Combination treatments and subsequent calculation of synergy were carried out according to the method from Chou and Talalay, with the compusyn software, available at http://www.combosyn.com/index.html. We have taken the mean of the raw CI values for the different concentration combinations, in order to determine it as overall synergistic (CI value < 0.9) or overall not synergistic (CI value > 0.9).

Proliferation assay

5×10^4 cells/ml were seeded per T75 flask. After 24 hours, 72 hours, 144 hours and 240 hours the cells were counted. From the linear growth fase, the doubling time was calculated with http://www.doublingtime.com/compute.php.

Westernblot

Total protein was collected by washing cells twice with ice cold PBS and subsequent lysis in RIPA buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100 (Sigma), 137 mM NaCl, 10% glycerol and protease inhibitors (Roche)). Concentration of the protein was measured with the Bio-Rad Dc Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. SDS-Page was used to separate the proteins, after which they were transferred onto a nitrocellulose membrane. Membranes were probed with a rabbit anti-pERK antibody (Cell Signaling, product nr #4376S), a rabbit anti-ERK antibody (Cell Signalling, product nr#9102) and a rabbit anti-GAPDH antibody (Abcam, Cambridge, UK, product nr ab9385), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, product nr sc-2030)Bound antibodies were detected using chemiluminescence (ECL, GE Healthcare, Chalfont St. Giles, UK). Afterwards, band intensity was measured using ImageJ software (imagej.nih.gov/ij/) and pERK band intensity was corrected for corresponding GAPDH band intensity.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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8. Manuscript 1 in preparation: IRS1 is not a general mechanism of MEK inhibitor resistance in NRAS mutated melanoma

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In preparation

IRS1 is not a general mechanism of MEK inhibitor resistance in NRAS mutated melanoma

Abstract

NRAS mutated melanoma patients quickly develop resistance to MEK inhibitor therapy, which is the only targeted therapy that is available for this patient group. Few other therapeutic options, besides immunotherapy, are available for these patients. As IRS1 was found to be a mechanism of BRAF inhibitor resistance in BRAF mutated melanomas, and inhibition of IRS1 resulted in cell death in these tumors, we wondered if IRS1 inhibition would also be a good option for NRAS mutated tumors. Therefore, we identified 18 NRAS-mutated melanoma cell cultures from our biobank, performed RNA-sequencing, and confirmed that 9 were resistant to MEK inhibition. High IRS1 expression was associated with MEK inhibitor resistance, although it was not a direct cause of resistance. This set included multiple paired cell culture, in which inhibition of IRS1 through siRNA knockdown or a small molecule targeting IRS1 of the top 5 high IRS1 expressing cells resulted in reduced viability in 1 cell culture, but not in a paired low-IRS1 expressing MEKi-sensitive cell culture from the same patient. We found that this reduced viability was independent of the PI3K-AKT pathway. Furthermore, this resistant cell culture had an MEKi dependent upregulation of pAKT, however, inhibition of pAKT via PI3K or AKT inhibitors did not reduce viability.

Introduction

Around 15-20% of melanoma patients have activating NRAS mutations. RAS is a GDP/GTP regulated kinase, which switches from an inactive, GDP bound state to an active, GTP bound state. In mutated forms of RAS, the protein is kept in a GTP-bound active state [1-5]. Other RAS isoforms besides NRAS are HRAS,KRAS4A, and KRAS4B, with each being expressed in different tissues and in various stages of development, while NRAS is the most frequent RAS isoform in melanoma [6].

Unlike the successful targeting of BRAF mutated tumors with BRAF inhibitors, efforts over the past 30 years to generate specific RAS inhibitors have failed. The only way to target RAS mutated tumors to date, is by inhibiting downstream effectors, which in the case of NRAS mutated melanoma is inhibiting the MAPK pathway by MEK inhibitors (MEKi). Unfortunately, NRAS mutated melanoma patients treated with MEKi rapidly develop therapeutic resistance. Resistance mechanisms to MEKi therapy have not been as extensively investigated as resistance to BRAF inhibitor therapy, but a likely possibility is that mutated NRAS can signal through effector pathways other than the MAPK kinase pathway.

Downstream signaling effectors of RAS, which play a role in the oncogenesis induced by constitutively active RAS, include RAF in the MAPK pathway [7], PI3K in the PI3K-AKT pathway [8] and RalGEFs in the RalGEF-Ral cascade [9-11]. Other effectors with less well studied roles in oncogenesis are the RacGEF Tiam1 [12], PLCε [13] and members of the RASSF family, which have a role in RAS mediated apoptosis [14].

. IRS1 is a signaling adapter molecule that transmits signals from IGFR to downstream signaling pathways, mainly the PI3K-AKT pathway and the MAPK pathway. Upregulation of IRS1 was found to be a mechanism of resistance for BRAF mutated tumors treated with BRAF inhibitors, and targeting IRS1 in these cell lines with small molecules resulted in significant cell death [15].We wondered if IRS1 inhibition could be a potential target for NRAS mutated melanoma and therefore we identified 18 NRAS mutated cell cultures from our biobank, which included patients who had been treated and those who had not been treated with a MEK inhibitor. We performed RNA-sequencing of these cells and tested their sensitivity to MEK162 treatment. We found that high IRS1 expression was associated with MEK162 resistance *in vitro*. However, in general siRNA mediated knockdown of IRS1 in the resistant cell cultures and transient overexpression of IRS1 in the sensitive cultures did not induce sensitivity or resistance, respectively, for MEK162 treatment, indicating that high IRS1 expression is not a direct cause of resistance in most of these cells. However, in the cell culture with the highest expression of IRS1, and independent of MEK162 treatment, siRNA mediated knockdown of IRS1 decreased viability. We therefore tested the drug NT157, which has been published to inhibit IRS1, and we found that this high-IRS1 expressing cell culture was also sensitive to NT157. We found that NT157 targets IRS1 through phosphorylation of S1101 and S612, but this does not result in inhibition of the PI3K-AKT pathway, which is the best studied downstream effector pathway of IRS1. In order to investigate the role of IRS1 in MEKi resistance, we examined other NRAS-mutated, low-IRS1 expressing melanoma cells derived from the same patient as the high-expressing cultures. We found that all of these cell cultures were sensitive to MEKi therapy, had low levels of IRS1 expression, and were resistant to NT157 treatment. Co-treatment of NT157 with MEK162 was antagonistic, which underscored the fact that a thorough understanding of the action of each drug and the interaction of different drugs is vital in order to make rational combinations that might ultimately benefit a patient.

Results

High IRS1 expressing melanoma cells are resistant to MEK-inhibitor treatment

We generated and analyzed 18 NRAS-mutated cell cultures from consenting patients treated or not treated with MEK inhibitor therapy. We found that 9 cell cultures were sensitive (i.e. IC50 < 100 nM MEK162) and 9 cultures were resistant (i.e. IC50>100 nM MEK162), as determined by in vitro cell viability assays. We performed RNA sequencing of these cultures and clustered them for *in vitro* MEKi sensitivity or resistance (Figure 1). We found high IRS1 expression was associated with *in vitro* MEK162 resistance and that the cell cultures with the highest IRS1 expression were resistant to MEK162 (Figure 2A).

siRNA mediated knockdown of IRS1 or treatment with NT157 decreases viability in one of the five highest IRS1 expressing cells

We went on to check the dependency of IRS1 in the 5 cell cultures with the highest IRS1 expression, as well as to test if high IRS1 expression was a direct cause of MEK162 resistance, by siRNA mediated knockdown of IRS1. We found that knockdown of IRS1 does decrease viability in the cell culture with the highest IRS1 expression (Figure 2B), but not in the other 4 cell cultures. For none of the cell cultures did IRS1 knock-down sensitize the cells to MEK162 treatment (Figure 2C). We next tested if

introducing IRS1 in a MEK162 sensitive cell culture with low expression of IRS1 can induce resistance to MEK162 treatment. We transfected M130429 (a MEK162 sensitive cell culture obtained from the same patient as M130219) with a transient GFP tagged IRS1 overexpression construct. We found that overexpression of IRS1 does not lead to MEK162 resistance in this cell culture (data not shown). We went on to test if the cell cultures with high IRS1 expression are sensitive to treatment with NT157, which is an IRS1 inhibitor that works by ERK dependent degradation of IRS1 following increased inhibitory Serine phosphorylation of IRS1. Consistent with the siRNA results, we found that the cell culture with the highest IRS1 expression, M130219, was sensitive to NT157, whereas the other 4 cell cultures were resistant (Figure 2D).

IRS1-mediated MEKi resistance in M130219 melanoma cells is independent of PI3K-AKT signaling

We next tested if high IRS1 expression led to increased PI3K-AKT signaling in the 5 high IRS1 expressing cells compared to a cell culture with low IRS1 expression. IRS1 expression was not associated with AKT pathway activity (Figure 3A). We next evaluated the phosphorylation of IRS1 Tyr612 in the cell cultures, as this phosphorylation site resembles active IRS1, which signals through PI3K-AKT. We only found this site to be phosphorylated in cell culture MM070221; however, this cell culture did not have high phosphorylation of AKT (Figure 3A and 3B). Furthermore, we found that the cell culture M130219, which was sensitive to NT157 treatment, was resistant for AKT inhibition and intermediately resistant to PI3K inhibition (Figure 5C). In addition, we noticed that administration of a PI3K inhibitor to M130219 completely prevented phosphorylation of AKT, whereas inhibition of IRS1 with NT157, which would be upstream of PI3K, does not prevent the phosphorylation of AKT (Figure 3C). Similarly, administration of Rapamycin led to increased phosphorylation of AKT through a positive feedback loop by which inhibition of mTOR by rapamycin leads to an inhibition of S6K, which results in activation of IRS1 and PI3K (by a reduction of the normal inhibitory effect of S6K on IRS) [16]. Combination treatment of Rapamycin and a PI3K inhibitor could prevent the phosphorylation of AKT, however, combination treatment of Rapamycin with NT157 was not able to block the phosphorylation of AKT (Figure 3C). As a final proof that NT157 did not interfere with PI3K-AKT signaling, we treated M130219 cells with soluble IGF1, which is a substrate for the IGFR, and we observed an increase in phosphorylated AKT at 20 min and 1 hour after IGF1 addition. This was seen to the same extent in the control and in the NT157 treated cells, whereas in the IRS1 siRNA knockdown cells this increase in AKT phosphorylation was inhibited (Figure 3D).

IRS1 blockade increases inhibitory Ser phosphorylation of IRS1 in M130219 melanoma cells and not STAT3 inhibition

We next analyzed the effect of NT157 on IRS1 and observed a dose dependent increase in inhibitory Ser phosphorylation of IRS1 upon NT157 treatment as previously described [15]; however, we did not observe a subsequent degradation of IRS1 protein (Figure 4A).

In a recent publication, it was shown that STAT3 is also a target of NT157, in which NT157 decreases phosphorylation of STAT3. When we compared STAT3 expression in our cohort of RNA sequenced cell lines, the NT157-sensitive culture M130219 had an average expression of STAT3. Furthermore, there was no difference in STAT3 expression between the cohort of NRAS-mutated, high-IRS1 expressing cells that were resistant to NT157 treatment and the cell line M130219 which was sensitive to NT157. When we treated M130219 with NT157, we could not see a reduction in STAT3

phosphorylation (Figure 4B), nor did treating M130219 with a STAT3 inhibitor result in decreased viability, as compared to cell cultures that were resistant to NT157 treatment (Figure 4C). Thus, we concluded that the sensitivity of M130219 to NT157 was not dependent on STAT3 inhibition.

Different cell cultures retrieved from different tumor sites from the same MEK162 resistant patient showed different responses on MAPK pathway inhibitor therapy and PI3K pathway inhibitor therapy From the same MEK inhibitor resistant patient where M130219 was derived from, we had generated 9 cell cultures from 9 different biopsies from multiple tumor locations during MEK162 treatment (M130219) and at autopsy post-resistance (M130420, M140404, M130421, M130428, M130425, M130429, M130427 and M130426) (Figure 5 A and B). We decided to study those in more detail in order to find a possible mechanism for MEK162 resistance as well as to see whether NT157 treatment would have likely benefitted this patient. We found that, *in vitro*, all the other cell cultures obtained at autopsy were sensitive for MEK and ERK inhibition. The different cell cultures also showed heterogeneity in their response on inhibitors of the PI3K pathway (Figure 5C). Notably, all samples were resistant for NT157, apart from M130219 (Figure 5D). When we treated the cell cultures with a combination of NT157 and MEK162, we found that these drugs antagonize each other, which is unwanted. Triple therapy with NT157, MEK162 and a PI3K inhibitor could prevent this antagonistic effect (Figure 5E).

Whole exome sequencing and RNA sequencing does not identify known genetic resistance mechanisms or substantial differences between the different cell lines

We next analyzed the genomic mutations in M130219 and the other cell cultures from the same patient by whole exome sequencing. In all cell cultures we found the following melanoma driver mutations: NRAS Q61R, MITF E318K and a CDKN2A loss. In total, 152 mutations were identified, which had more than 10x coverage for that coordinate across all samples including the germline, for which two or more variant callers supported it (of mutect, varscan, multiSNV and indelgenotyper), that didn't overlap with repetitive regions and that were not present in the 1000 genome project or EVS databases. Of these 152 mutations, only two genes were found to be mutated in M130219 and not in the other cell cultures: SOS2 and GCOM1. However, these mutations we could not find in the RNA sequencing, suggesting that the mutant allele was not expressed.

The resistant cell culture M130219 has a mesenchymal transcription profile and an upregulation of multiple RTKs compared to the sensitive cell cultures

In addition to the difference of IRS1 expression in M130219 compared to the others (Figure), we looked for other transcriptional differences between the cell cultures that could explain the resistance to MAPK pathway inhibitors of M130219. By geneset enrichment analysis, we found that M130219 has a more mesenchymal signature compared to the sensitive cell cultures, which are more melanocytic (suppl. Table 1). Furthermore, we found that M130219 has multiple RTKs upregulated, however, M130219 was resistant to inhibitors targeting those RTKs (Suppl figure 1).

Discussion

We have shown that high IRS1 expression is associated with MEK162 resistance in the 18 NRAS mutated melanoma cell cultures we have tested. However, based on experiments with siRNA

mediated knockdown of IRS1, and unlike in BRAF-inhibitor resistant cells, we conclude that IRS1 is not generally sufficient for MEK162 resistance. Nevertheless, in the cell culture with the highest IRS1 expression, knockdown of IRS1 independent of MEK162 treatment caused a decrease in cell viability, suggesting that this cell culture is dependent on IRS1 expression and/or the pathway through which IRS1 is signaling. However, this pathway is not the usual IRS1 target PI3K-AKT pathway, as the basal phosphorylation of AKT was very low in this cell culture, as well as IRS1 pTyr612, suggesting a low activity of the PI3K pathway. Treatment with a PI3K or AKT inhibitor did not decrease viability. Consistent with the siRNA data, treatment with NT157, an IRS1 inhibitor, decreased viability in the high-IRS1 expressing cell culture which also was responsive to siRNA mediated knockdown, but not in the other melanoma cultures. Furthermore, NT157 acts on IRS1, as indicated by the increased Serine phosphorylation upon treatment. NT157 did not act via the PI3K-AKT pathway, as stimulation with IGF1 during NT157 treatment still increased phosphorylation of AKT. These findings are consistent with Song et al, who showed that IRS1 phosphorylation at Serine 1101 does not interfere with IRS1 signaling via the PI3K-AKT pathway [17].

Although both the knockdown and inhibitor experiments suggest that IRS1 is playing an important role in MEKi-resistance in M130219 cells, the pathway or mechanism in which IRS1 exerts is effect needs further investigation. Known IRS1 targets other than PI3K include SHP-2 [18], Grb-2 [19, 20], Fyn [21], Nck [22] and Crk [23], but, these downstream effectors are typically recruited by IRS1 phosphorylation on tyrosine residues. On the contrary, increased phosphorylation of serine residues on IRS1 has been associated with facilitation of interaction with integrins [24, 25]. In neurons, increased serine phosphorylation of IRS1 upon TNFa treatment leads to increased binding of integrin b1 to IRS1, which results in reduced development and stability of neuronal processes and reduced attachment to collagen IV [25]. From the 18 NRAS mutated cell cultures that were analyzed by RNA-seq, M130219 had the highest expression of ITGA1, ITGA11, ITGA8 and ITGB2. However, a scenario where increased IRS1 Serine phosphorylation upon NT157 treatment leads to increased integrin binding, thereby reducing cell viability, cannot explain why reduced cell viability is also observed in siRNA mediated knockdown of IRS1. Nevertheless, it would be interesting to test the effect of TNFa on M130219 as well as perform 3D spheroid cultures and invasion assays under NT157 treatment, to see if integrins play a role in M130219.

Especially Serine 1101 is hyper-phosphorylated upon NT157 treatment, Song et al has shown that Pim-kinase is responsible for the phosphorylation of IRS1 Serine 1101 and that a pan-PIM inhibitor inhibits this phosphorylation [17]. It would be interesting to see if treatment with NT157 leads to an increased expression of Pim, which could explain the increased phosphorylation at Serine 1101 upon NT157 treatment. Furthermore, in order to test whether this phosphorylation is the mode of action of NT157, one could do combination treatment with NT157 and a pan-PIM inhibitor.

Serine 1101 is positioned in the SHP2 domain of IRS1 [26]. SHP2 is a tyrosine phosphatase and Luo et al has shown that phosphorylation of IRS1 on S1223, which is also in the SHP2 domain, interferes with the recruitment of SHP2, leading to increased tyrosine phosphorylation of IRS1 [27]. We have evaluated the phosphorylation of Tyrosine 612, located in the PI3K domain, which was low at baseline and unaltered upon NT157 treatment. However, other tyrosine phosphorylation sites recruiting other effectors may be altered and yield important information of the mode of action of NT157.

Material and Methods

Patient and sample preparation

Surplus material, collected during or after therapy and at autopsy, was used from patients who had given written consent according to ethical approval numbers 647 and 800. Primary cell cultures were generated and kept under standard conditions (i.e. without drug treatment) as described in [28].

Cell viability

Cell viability was measured with the assay described in [29]. Drugs tested in different doses were those targeting the MAPK pathway (MEK (MEK162) and ERK (SCH772984) inhibitors) and the PI3K pathway (PI3K (GDC-0941), AKT (GSK690693) and mTOR (Rapamycin) inhibitors), as well as the IRS1 inhibitor NT157.

DNA preparation and Exome sequencing

From all cell cultures, DNA was prepared using QiAmp DNA blood mini kit (Qiagen), according to the manufacturers' instructions. Library preparation, sequencing and subsequent analysis were done as described in [29].

RNA preparation and RNA sequencing

RNA was isolated with the QIAGEN RNeasy Mini kit (74104, QIAGEN, Venlo, Netherlands). RNA-seq libraries were prepared using poly(A) enrichment and sequenced on a HiSeq4000. Raw reads were aligned to hg38 and gene counts with gencode v22 using STAR [30] aligner. Gene counts were normalized with edgeR [31] and differential expression performed with limma voom [32].

Westernblot

Protein isolation, sample preparation and westernblot was done as described in (cite reference 2). Membranes were probed with a rabbit total IRS1 antibody (Sigma Alderich), a rabbit anti-pERK antibody (Cell signaling, product #4376), a rabbit anti-pAKT Ser473 antibody (Cell signaling, product #9271), different rabbit IRS1 antibodies (from the IRS-1 inhibition antibody sampler kit, cell signaling, product #12879) , a rabbit pSTAT3 Tyr705 antibody (cell signaling, product #9131) and a rabbit anti-GAPDH antibody (Cell signaling, product #2118), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, product sc-2030). As a detection reagent ECL (..) was used and blots were developed using .

siRNA of IRS1

Cells were seeded at 90% confluency in T25 cell culture flasks (Falcon). The following day, the medium in the flasks was changed to DMEM with 10% FCS and cells were transfected with a mixture of DMEM, interferrin and a final concentration of 20 nM siRNA IRS1 or siRNA allstars negative control. After 24 hours, the transfection medium was removed and cells were trypsinized and seeded for the subsequent experiments.

Transient overexpression of IRS1

Cells were seeded in 90% confluency in T75 cell culture flask (Falcon). The following day, medium was refreshed to DMEM with 10% FCS and cells were transfected using a mixture of . After 6 hours of transfection, medium was refreshed to standard culture medium (reference 1). 48 hours post transfection, cells were sorted, using the FACS… sorter on GFP positive and GFP negative cells. These cells were seeded for subsequent experiments.

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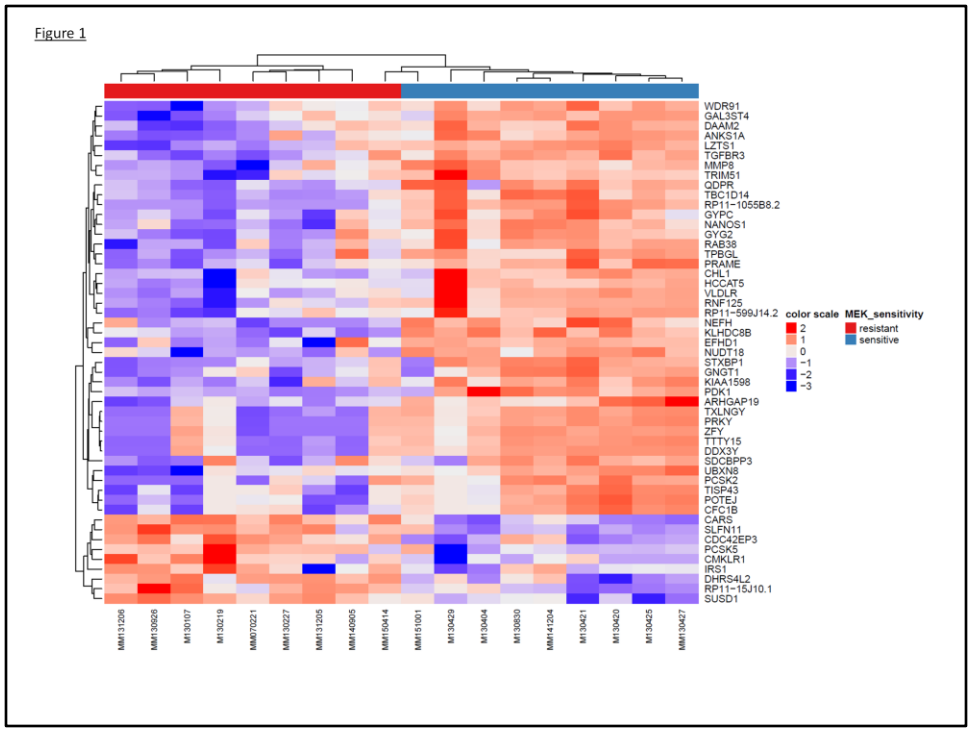

Figure 1: Heatmap clustered for MEK inhibitor sensitive and resistant cells
Shown are the top 50 differentially expressed genes, including IRS1. The top 50 fits into FDR>0.2

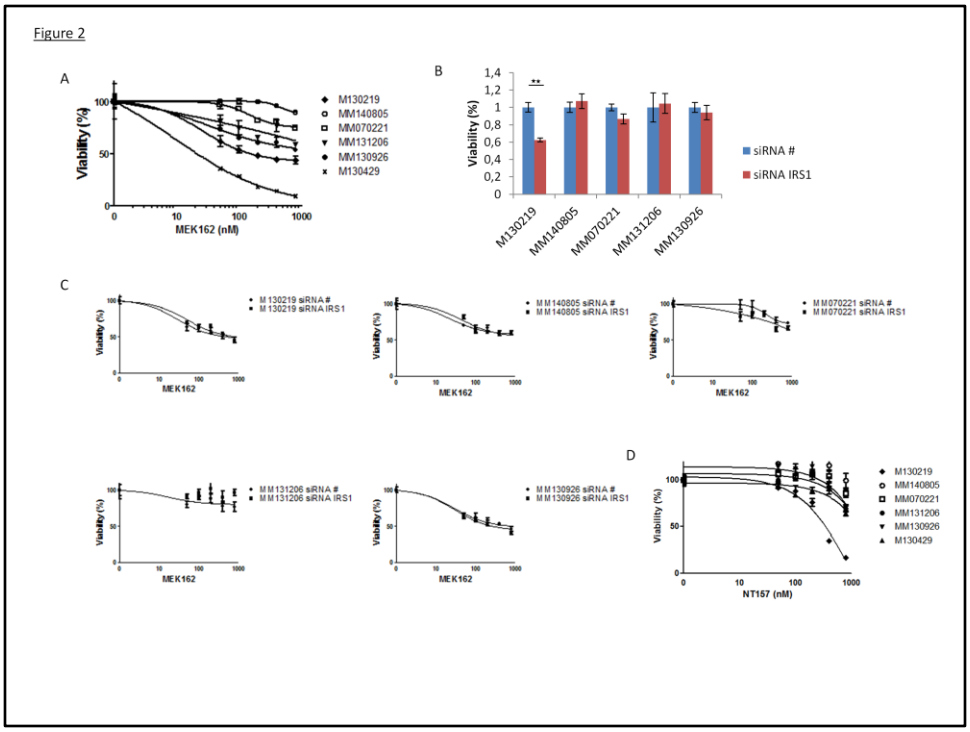

Figure 2: high IRS1 expression correlates with MEK162 resistance in NRAS mutated melanoma but is not a direct cause of resistance

A: Viability under MEK162 resistance of the top 5 high IRS1 expressing NRAS mutated melanoma cells compared to a MEK162 sensitive cell culture with low IRS1 expression (M130429)

B: Viability of high IRS1 expressing cells with siRNA mediated IRS1 knockdown

C: Viability of high IRS1 expressing cells with siRNA mediated IRS1 knockdown under MEK162 treatment

D: Viability of high IRS1 expressing cells under NT157 (IRS1 inhibitor) treatment

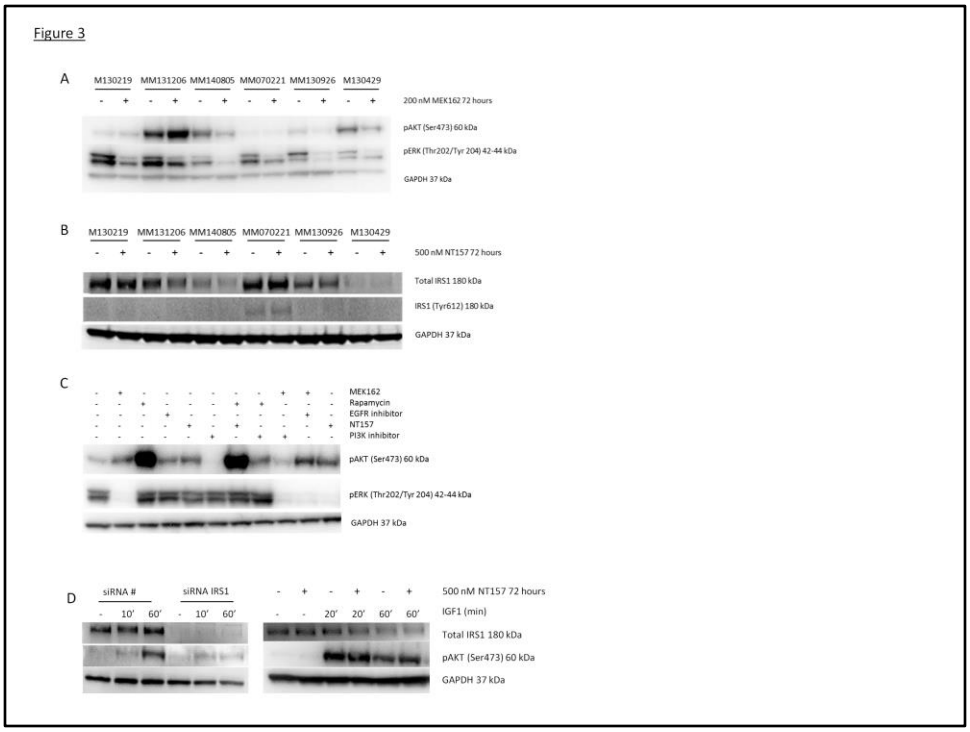

Figure 3: The dependency of M13019 on IRS1 is not mediated by signaling through the PI3K-AKT pathway

A: Westernblot showing the expression of pAKT and pERK under standard conditions and under MEK162 treatment in high IRS1 expressing NRAS mutated melanoma cells

B: Westernblot showing the expression of total IRS1 and of active IRS1 Tyr612 in high IRS1 expressing NRAS mutated melanoma cells

C: Westernblot showing pAKT and pERK under various treatment conditions in M130219

D: Westernblot showing total IRS1 and pAKT under IGF1 treatment in NT157 treated compared to siRNA mediated IRS1 knockdown treated M130219

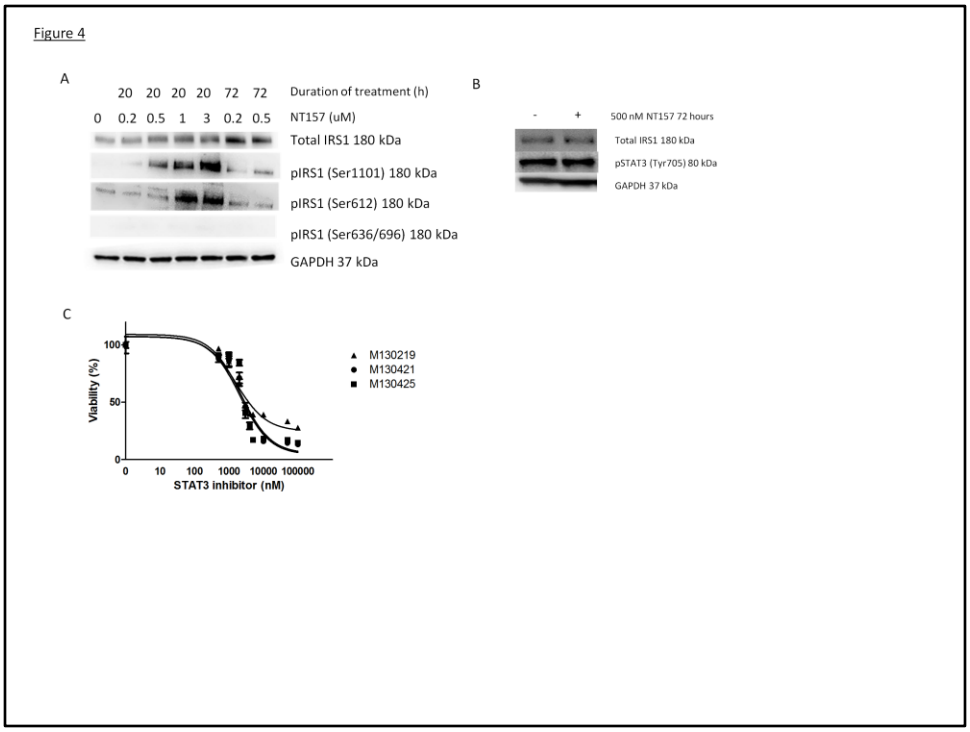

Figure 4: NT157 increases Ser phosphorylation of IRS1 and does not have an effect on pSTAT3 in M130219
A: Westernblot showing Ser1101, Ser612 and Ser636/696 phosphorylation of IRS1 under various dosages and timepoints of NT157 treatment
B: Westernblot showing pSTAT3 under NT157 treatment in M130219
C: Viability of M130219 compared to two low IRS1 expressing, NT157 resistant cell cultures (M130421 and M130425) under STAT3 inhibitor treatment

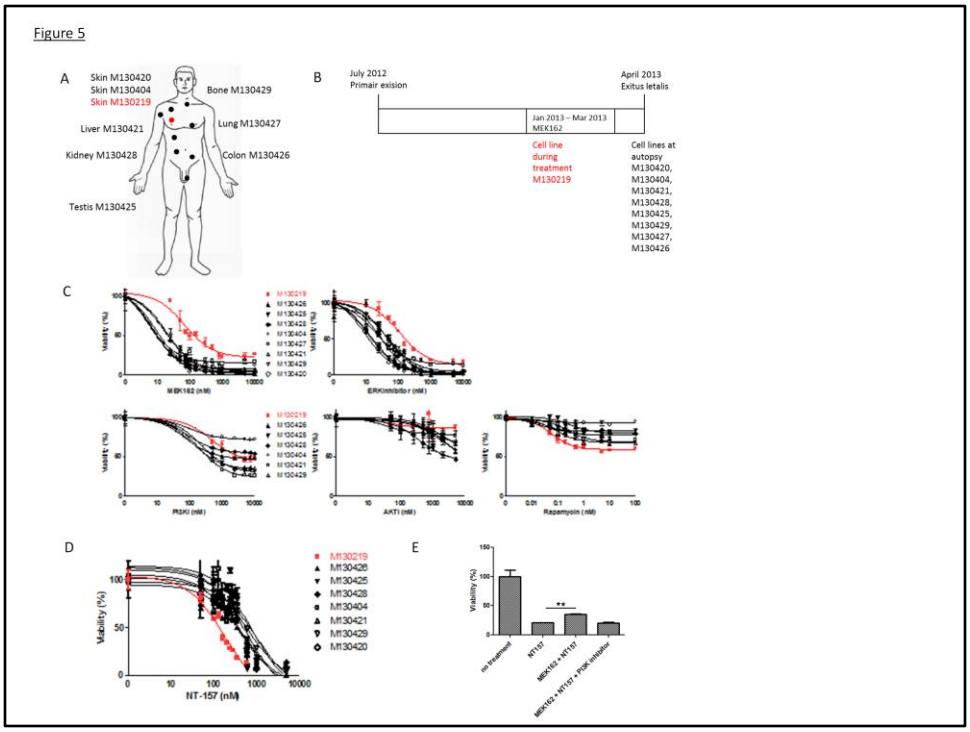

Figure 5: Different cell cultures retrieved from different tumor sites from the same MEK162 resistant patient showed different responses on MAPK pathway inhibitor therapy and PI3K pathway inhibitor therapy

A and B: Multiple cell cultures were generated from different tumor sites from the same MEK162 resistant, NRAS mutated patient

C: Viability assays of different MAPK and PI3K-AKT pathway inhibitors on different cell cultures from the same patient

D: Viability assay of different cells cultures treated with NT157

E: Viability of M130219 under different combinations of drugs

9. Review 1: Melanoma immunotherapy: historical precedents, recent successes and future prospects

Melanoma immunotherapy: historical precedents, recent successes and future prospects

The idea of cancer immunotherapy has been around for more than a century; however, the first immunotherapeutic ipilimumab, an anti-CTLA-4 antibody, has only recently been approved by the US FDA for melanoma. With an increasing understanding of the immune response, it is expected that more therapies will follow. This review aims to provide a general overview of immunotherapy in melanoma. We first explain the development of cancer immunotherapy more than a century ago and the general opinions about it over time. This is followed by a general overview of the immune reaction in order to give insight into the possible targets for therapy. Finally, we will discuss the current therapies for melanoma, their shortcomings and why it is important to develop patient stratification criteria. We conclude with an overview of recent discoveries and possible future therapies.

KEYWORDS: cancer immunotherapy · cancer testis antigens · Canvaxin™ · CTLA-4 · immunotherapy · interferon · melanoma · PD-1 · T-cell therapy · vaccines

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History of cancer immunotherapy

In 1890, a surgeon in New York City, named William Coley, directly injected streptococcal bacteria into the inoperable tumors of cancer patients in order to induce an immune response to combat tumors. He had some notable successes in regressing tumors, which were published in 1893 [1–3]. This was the first demonstration in medical literature that the immune system could be used to kill cancer cells and was subsequently applied to hundreds of cancer patients by Coley for the rest of his career.

However, during this time, immunotherapy was not considered to be a serious cancer therapy, as Coley's successes were sporadic, difficult to reproduce and lacked a solid theoretical foundation. In a major review published in 1929, WH Woglom even wrote that "it would be as difficult to reject the right ear and leave the left ear intact as it is to immunize against cancer" [4]. Further rejection of cancer immunotherapy came following the discovery that, during the development of the immune system in prenatal life, lymphocytes that react to self-tissues are destroyed [5]. Therefore, the combination of unconvincing clinical data and a general paradigm in which immune cells were incapable of recognizing any self-tissue prevented progress in cancer immunotherapy for several decades.

A few exceptions to this trend occurred in the 1950s, when tumors were discovered to be recognized by the immune system [6–9] and in the 1960s, when Burnet proposed that lymphocytes continuously checked tissues for transformed cells to destroy, probably through the recognition of tumor-associated antigens [10]. A similar theory had already been proposed in 1909 by Ehrlich [11]; however, the theory did not receive much support, as several experiments (of which later the validity was questioned) showed contradictory evidence.

Thus, until the 1980s, cancer immunotherapy was considered to be ineffective and irreproducible.

However, three discoveries in the mid-1990s changed the prospects of cancer immunotherapy. First, it became clear that some autoreactive T cells can be found in the blood, which theoretically could be directed against transformed self-cells [12–14]. Second, immunogenic cancer antigens were discovered [15–19], suggesting that they may be recognized and cleared by the immune system. Finally, malignant cells were shown to be highly genetically unstable [20,21]. This genetic instability could produce cancer-specific epitopes on the cell surface that might distinguish cancerous from normal cells.

Most convincingly, in 1995 it was demonstrated that dendritic cells can induce tumor-specific T-cell immunity and regression of melanoma metastases when appropriately activated and reinjected [22–25]. Lastly, immunocompromised mice were observed to have a higher incidence of carcinogen-induced tumors [26]. This, together with further proof that both the adaptive and innate immune systems play a role in eliminating tumors [27], has put immunotherapy back on the list of potential anticancer therapies.

Since the early 1990s, researchers have developed several ways to stimulate the patient's

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immune system, some of which were successful and some less so, depending on the cancer type (for a historical overview see [28]). Melanoma in particular is a highly immunogenic tumor, as was confirmed in 1999 after a study identified circulating T cells that could specifically recognize tumor-associated antigens on melanoma cells [29]. Therefore, many attempts to induce an immune response against cancer have been tested in melanoma. An extensive overview of these studies can be found in the review from Zeiser *et al.* [30].

The immune reaction

In order to induce an antitumor immune response, specialized APCs, called dendritic cells (DCs), have to process antigens from the tumor and present them to naive T cells **(Figure 1)**. Products of mutated or nonmutated genes expressed specifically by tumors (e.g., cancer-testis antigens [CT-antigens]), as well as differentiation antigens specific for the cancer's tissue of origin (e.g., melanocytic differentiation antigens) can all serve as tumor antigens [31]. Each type of antigen has advantages and disadvantages. Products of mutated genes will be exclusively expressed by tumor cells, therefore reducing the risk of autoimmunity; however, as they are also patient specific, they cannot be translated to a broader group and may exhibit a high degree of heterogeneity within each patient. CT-antigens, on the other hand, are normally only expressed in the testis, where the blood–testis barrier prevents the entrance of immunologic cells [32] and are re-expressed in many cancers. Hence, the presence of T cells directed against CT-antigens will not induce autoimmunity, since these genes are not normally expressed in adult somatic tissues. One example of CT-antigens used in cancer therapy for melanoma and ovarian cancer showed that vaccination for NY-ESO-1 resulted in the generation of T cells directed against NY-ESO-1, with a favorable clinical response in some patients [33]. Melanocytic differentiation antigens are also expressed by normal melanocytes; therefore vaccination with these antigens could lead to autoimmune-induced pigmentation loss (vitiligo) [34]. DCs can directly obtain and process antigens from the tumor as demonstrated by the discovery of tumor-infiltrating T cells (TILs) in some cancer patients (i.e., those with a better prognosis) [35]. The antigens could also be delivered via a therapeutic vaccine. These antigens can be in the form of synthetic peptides or proteins, tumor-cell lysates, or antigen-encoding DNA or RNA in viral vectors.

In order to be activated upon capture and antigen presentation, DCs must receive an immunogenic maturation stimulus, such as microbial peptides or proinflammatory cytokines; without such a stimulus, an opposite reaction will induce tolerance by T-cell deletion and/or the production of Tregs [36–39]. Immunogenic maturation signals not only can be derived from necrotic tumor cells, but also can be therapeutically administered. DC maturation is induced by activated pattern recognition receptors such as Toll-like receptors, therefore, Toll-like receptor ligands or agonist antibodies may be used to stimulate the DCs. Such stimulated DCs will process the captured antigen and present it on MHC class II molecules, at which point they are transported to the draining lymph node, interact with T cells and induce an immune response.

Effective interaction of the MHC/antigen molecule with the T-cell receptor (TCR) requires a costimulatory signal in the form of either plasma membrane ligands on the DC that interacts with stimulatory or inhibitory receptors on the T cell, or in the form of secreted cytokines [40]. Several costimulatory and inhibitory molecules have been identified, of which CD28 has received the most attention. CD28 encodes the primary costimulatory receptor expressed on T cells that is required for a rapid T-cell response. Recent research on the therapeutic potential of this protein is a cautionary tale of how direct modulation of the immune response can have dramatic and unexpected consequences in human patients. A CD28 superagonist immunomodulatory drug (TGN1412) that was developed to activate the immune response in B-cell chronic lymphocytic leukemia patients resulted in a massive and potentially lethal cytokine storm within 12–16 h after administration of the drug in human volunteers and the trial was immediately terminated [41]. The unexpected and severe reaction to this CD28 agonist suggests that therapeutics designed to harness the immune response to fight cancer must proceed with extreme caution in order to ensure controlled and specific targeting of cancer cells.

Not only must effective cancer immunotherapies be predicated on a thorough understanding of normal immune functions, but the mechanisms that cancer cells employ to evade immunoregulation may also provide clues as to how to better modulate the immune response.

In cancer, after the T cells have been activated they enter the tumor bed to destroy the antigen-expressing cancer cells. Tumors, however,

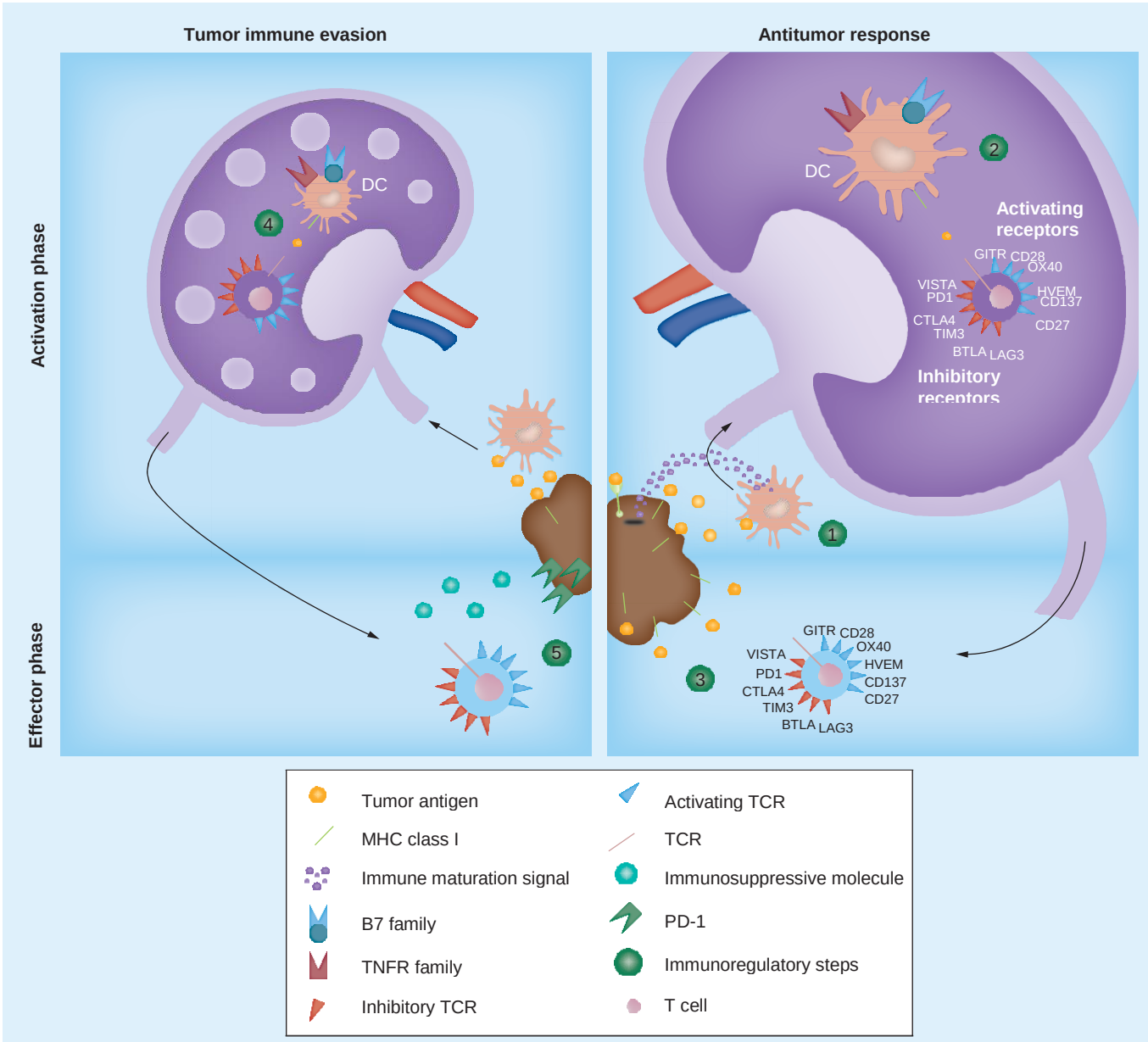

Figure 1. Tumor evasion mechanisms and immune reaction against tumors. The immune reaction that leads to tumor elimination is shown on the right. In the activation phase (upper-right corner), tumor antigens expressed by the tumor are taken up by DCs (step 1). The DCs will be activated by an immune maturation signal and travel to the lymph node. Here they present the antigen on MHC class II molecules to naive T cells (step 2). These T cells will become activated if a costimulatory signal is received. This costimulatory signal can be in the form of interaction with plasma membrane ligands of the TNF family or the B7 family on the dendritic cell with activating receptors on the T cells. Both the TNF and the B7 family of ligands interact with activating receptors, whereas only B7 family ligands interact with inhibitory receptors. When the activated T cell travels to the tumor (lower-right corner; effector phase) it will recognize the antigens and eliminate the tumor (step 3). However, the tumor possesses some defense mechanisms (shown on the left). In the activation phase, tumors can downregulate their antigen expression by downregulation of MHC class I molecules. Also, the immune maturation signal can be absent, which will lead to tolerance to the antigen, as interaction of the unactivated DC with the naive T cell in the lymph node will induce the formation of Tregs (step 4). In the effector phase, tumors can overexpress inhibitory ligands, such as PD-1 ligand, which will interact with inhibitory receptors on T cells. Furthermore, tumors can secrete immunosuppressive molecules such as PGE2, arginase, IDO and VEGF, which will prevent the T cells from eliminating the tumor (step 5).
DC: Dendritic cell; TCR: T-cell receptor; TNFR: TNF receptor.

possess a large battery of defense mechanisms [42] and the tumor microenvironment may also have a suppressive effect on tumor-infiltrating lymphocytes. This is demonstrated by the fact that these cells are unable to destroy tumor cells *in vivo*; however they do have immunologic

properties when they are removed from the tumor environment [43]. The tumor defense mechanisms include the presence of Tregs and myeloid-derived suppressor cells in tumor tissue, secretion of T-cell-suppressive factors such as IL-6, IL-10, VEGF and TGFβ, as well as the (over)expression of ligands for inhibitory receptors on T cells. In general, an immune response can be divided into an activation phase, consisting of the activation of DCs and their interaction with T cells in the lymph node, and an effector phase, consisting of the actual invasion of activated and selected T cells in the tissue, and the elimination of cells expressing the antigen (Figure 1). In both phases, immunological checkpoints prevent the reaction against 'self' cells. Tumor cells can use these checkpoints as a defense mechanism by not letting the immune reaction pass the checkpoint. Many checkpoints work via receptor–ligand interactions that are either stimulatory or inhibitory. Tumors can activate the inhibitory interaction by (over) expression of ligands for inhibitory receptors on T cells, for example PD-1 ligand [44].

Targets for therapy

Aside from strategies that directly regulate the immune system, the identification of tumor-specific antigenic targets has been challenging. Owing to cells within a tumor being genetically and behaviorally heterogeneous [45], a specific therapeutic target may not be expressed in all cancer cells. CT-antigens, for example, are only expressed in 5–50% of the tumor cells; therefore, it is unlikely that vaccination against these antigens will target all cancer cells [46]. Even if certain antigens such as melanocytic differentiation antigens are homogeneously expressed in tumor tissue [47], some cells may develop additional strategies to overcome the therapy, such as downregulating the targeted proteins. In addition, it is likely that immune tolerance mechanisms of the tumor work in combination with each other and, therefore, combination therapy allows for the simultaneous attack of multiple targets with likely synergistic effects.

Current treatment options

Despite some setbacks, cancer immunotherapy for melanoma has shown early moderate success with IL-2 and interferon, and more recent significant results through targeting the inhibitory receptor CTLA-4. In addition, inhibiting the interaction of PD-1 ligand with the PD-1 receptor, either by anti-PD-1-receptor or anti-PD-1-ligand antibodies, is a promising recent therapy [48,49].

IL2

More than three decades ago, IL-2 was shown to expand T cells with antitumor activity *in vitro* and *in vivo* [50]. Subsequently, treatment with high-dose (HD), single-agent IL-2 in metastatic melanoma and renal cell carcinoma patients was investigated, which demonstrated 7% complete remission (CR) and 10% partial remission in 134 melanoma patients [51]. A subsequent publication including 270 melanoma patients from multiple clinical studies reported CR in 17 patients (6%) and partial remission in 26 (10%)[52]. In the follow-up report in 2000, the authors estimated a CR median duration of at least 59 months, although this had not been reached in their patient population.

Based on available evidence, but without a randomized controlled trial, HD IL-2 was approved by the US FDA for the treatment of metastatic melanoma in 1998. However, the moderate results, as well as the drawbacks of IL-2, such as its toxicity profile and quality of life impairment, diminished its general applicability. Studies with low-dose IL-2 failed to show improvement over HD treatment [53]. Reviewing all clinical data available on IL-2 treatment in melanoma, there seems to be a mild benefit without improvement of overall survival (OS) [53]. Arguably, these outcomes are still comparable with dacarbazine (DTIC) or other chemotherapies. Fortunately, the toxicities caused by IL-2 therapy have become more predictable and manageable as experience with IL-2 has increased over the years. Moreover, the durable long-term disease-free survival recorded in selected patients still suggests IL-2 therapy as a possible treatment option for metastatic melanoma.

In order to improve the outcome with IL-2, many studies were conducted that investigated combinations of IL-2-based immunotherapy and chemotherapy, termed biochemotherapy [54]. The initial results of combined cisplatin-based chemotherapy and IL-2 versus single arms were promising [55,56]; however, two Phase III trials were unable to produce statistically significant response improvements and failed to show an OS benefit [57,58]. In another Phase II study, Tarhini *et al.* investigated sequential temozolomide followed by HD IL-2 in 38 patients [59]. Their results indicated that although this combination had lower toxicity than previous biochemotherapies, the overall response rate and durability of responses did not

exceed those of single-agent HD IL-2. High levels of VEGF and fibronectin were shown to correlate with lack of clinical response to IL-2 therapy and decreased OS [60].

Interferon

Interferons (IFNs) are pleiotropic cytokines that were first described in 1957 as proteins that interfere with viral replication [61]. IFNs induce synthesis of hundreds of different proteins by activating the JAK-STAT pathway, which is a major signaling system that cells use to transmit extracellular information from many cytokines and growth factors to the nucleus [62,63]. Seven IFNs have been described in humans and based on the type of receptor they bind to, all these IFNs fall into two main types produced by different cell populations [64]. Type I IFNs (IFN-α, IFN-β and IFN-ω) bind to IFN-α receptors, whereas type II IFNs (IFN-γ) bind to IFN-γ receptors. Although the different types of IFN have little structural similarity and may signal through distinct receptors and pathways, they also exhibit overlapping effects, particularly through their antiproliferative activities [62].

IFN treatment is the most studied and only approved adjuvant therapy for melanoma patients. IFN-α2b was f irst shown to be beneficial in stage II/III melanoma without sentinel node microstaging [64]. Other studies conducted in the 1990s also demonstrated that adjuvant IFN can significantly increase disease-free survival [65–68] and in some studies, overall survival [64,68,69,70]. Hence, IFN was the first agent to show a significant benefit in relapse-free survival and OS of high-risk melanoma patients in a randomized controlled trial [64]. These results finally led to FDA approval of HD IFN-α2b adjuvant therapy for intermediate- and high-risk melanoma patients. Low-dose conventional IFN-α, three milli-international units administered three-times weekly, is approved as the standard dosing for adjuvant IFN therapy by most European countries [71].

Currently, IFN is applied either in standard or pegylated form (PEG) and, until recently, the latter was only used for treatment of chronic hepatitis C. The pegylation of IFN-α by covalent binding of polyethylene glycol increases its half-life from 3–8 h to 22–60 h (PegIntron®, Schering-Plough, NJ, USA; IFN-α2b with 12-kDa PEG-chain) or even to 60–80 h (Pegasys®, F Hoffmann-La Roche, Basel, Switzerland; IFN-α2a with 40-kDa PEG-chain) without changing its tertiary structure or spectrum of activity [62]. The advantage of this is that it can be injected only once per week, whereas the standard IFN needs to be injected three-times per week. However, the use of IFN, both in its standard and pegylated form, is associated with an extensive range of side effects and must be carefully monitored [72].

Based on these results, the largest IFN adjuvant trial to date was initiated (i.e., the EORTC18991 trial), which included detailed information on microstaging and ulceration [73]. A total of 1256 patients with resected stage III melanoma were randomly assigned to observation (n = 629) or pegylated IFN-α2b (n = 627) 6 µg/kg per week for 8 weeks (induction), followed by 3 µg/kg per week (maintenance) for an intended duration of 5 years. The authors of this study concluded that patients with sentinel node involvement and ulcerated melanoma benefited the most, in terms of relapse-free survival, distant metastasis-free survival and OS [73].

Anti CTLA4 antibody

Activated T cells play an important role in mediating host immune response against tumors. Nevertheless, tumors are able to evade detection and destruction by the immune system through local and regional immunosuppressive mechanisms. Ipilimumab (Yervoy™, Bristol-Meyers Squibb, NY, USA) is a fully human monoclonal antibody that binds to and blocks CTLA-4. CTLA-4 is one of the inhibitory receptors on T cells that will inhibit T-cell activation when it binds to the B7-family proteins on the cell membrane of DCs (Figure 1) . Subsequently, blocking CTLA-4 with anti-CTLA-4 antibod- ies enhances T-cell responses *in vitro* and *in vivo*, and activates proliferation of tumor-specific T cells. The efficacy of ipilimumab was investi- gated in a large, randomized, Phase III clinical trial involving 676 patients with unresectable stage III or IV melanoma, whose disease had progressed during therapy for metastatic disease [74]. Patients were treated with ipilimumab plus the peptide vaccine gp100 (n = 403), ipilimumab alone (n = 137) or gp100 alone (n = 136). The aim of the study was to demonstrate the benefit of the combination of antibody and vaccination. Surprisingly, the results showed that the median OS was increased in all patients receiving ipilimumab from 6.4 months in the gp100 alone group to 10 months in the ipilimumab plus gp100 group and 10.1 months in the ipilimumab alone group. This positive impact on OS in patients with advanced melanoma has led to the approval by the FDA, EMA, SwissMedic, Health Canada and most

recently, by the National Institute for Health and Clinical Excellence for use in England and Wales. However, cost concerns and contradictory follow-up results have led to some concerns regarding the overall efficacy of this new treatment. For instance, in a recently published study of 82 patients, no association between the status of the well-known BRAF V600E mutation of melanoma and disease control after treatment with ipilimumab was detected [75]. Currently, no biomarkers are available to identify patients who will benefit from ipilimumab treatment. Another Phase III study investigating the use of ipilimumab in combination with DTIC in patients with previously untreated metastatic melanoma also reported prolonged OS compared with DTIC plus placebo treatment [76]. The combination of ipilimumab with DTIC leads to an increased hepatotoxicity [76]. Magolin *et al.* showed that the activity of ipilimumab in patients with melanoma brain metastasis is similar to patients with advanced melanoma without brain metastases [77]. Ipilimumab is associated with immune-related adverse events [74,78], requiring specific management. Autoimmunity and the appearance of thyroid dysfunction have already been reported with IL-2 and IFN-α [79], indicating a correlation between autoimmunity and antitumor response. The mode of action of immune-potentiating agents such as ipilimumab led to the formulation of novel radiologic tumor response criteria (immune-related response criteria) [80], since these agents can result in an initial increase in the size of a tumor followed by a regression. Using the common response evaluation criteria in solid tumors, a size increase or new tumor lesions correspond to 'progressive disease'. However, this initial increase in tumor size might be due to local inflammation, subsequent to recruitment of activated T cells at tumor sites [77,81].

Combination therapies of ipilimumab with the specific BRAF kinase inhibitor or with radiotherapy and other molecular targets are currently under investigation [201,202]. In addition, the results of a clinical trial analyzing the efficacy of ipilimumab as an adjuvant treatment in melanoma patients, along with another comparing two different dosages will give more information concerning the dosage and optimal time points of treatment [203].

Vaccines

Most people are familiar with the global success of vaccines in eradicating or controlling important diseases. However, with only a few notable successes, cancer vaccination has had little clinical benefit overall, especially for the treatment of metastatic melanoma [82,83]. Numerous clinical vaccination trials have been conducted, in particular with irradiated whole-tumor cells mixed with bacterial adjuvants such as Bacillus Calmette-Guérin or *Corynebacterium parvum*, showing small but significant effects in melanoma treatment [83,84]

One of the most ambitious whole-cell melanoma vaccines (Canvaxin™, CancerVax, DE, USA) is another cautionary tale on the road to developing a melanoma vaccination. This vaccine (developed from multiple melanoma cell lines) initially showed some promise in matched-pair analyses of patients receiving the vaccine after surgery; however, subsequent data showed the median survival of the placebo group to be longer than the vaccine arm [85–87]. A similar approach was taken by the manufacturers of Melacine® (Corixa, WA, USA), which also reached Phase III studies [88]. Once again, an initially auspicious outlook from Phase I and II trials led to optimism for the clinical application of Melacine, but Phase III trials failed to show any benefit in the total study group [89].

The development of a more comprehensive molecular understanding of melanoma progression and immune recognition in the last decade has led to more focused attention on specific melanoma antigens. A variety of tumor-associated antigens such as differentiation antigens, CT-antigens and viral antigens (as listed in the T-cell defined tumor antigen database at [204]) have been studied to augment antitumor immune responses in animal models and partly in clinical trials. The antigen-based vaccine therapies can be divided into:

- Plasmid DNA-based vaccines that deliver the gene encoding the antigen
- Recombinant viral and bacterial vaccines
- Peptide- or protein-based vaccines that deliver the antigen mixed with adjuvants
- Antigen-pulsed DC vaccines

DNA vaccines provide the opportunity to express the immunizing antigen over time as they are based on the ability of the cell to transcribe encoded genes from DNA episomes delivered to the host. The exact mechanism by which DNA vaccines induce antitumor immunity is yet to be determined; however, several studies suggest that immunity is generated by DNA transfection of muscle cells and keratinocytes [90]. One example is Allovectin-7® (Vical, CA, USA), a bicistronic

plasmid encoding human leukocyte antigen-B7 and β-2 microglobulin, an immunotherapeutic agent designed to express allogeneic MHC class I antigen upon intralesional administration. This vaccine seems to be an active, well-tolerated treatment for selected stage III/IV metastatic melanoma patients with injectable cutaneous, subcutaneous or nodal lesions [91], and is currently being studied in a Phase III clinical trial [205].

Several recombinant viral vectors are also under investigation to augment antitumor immune responses against model tumor antigens. Of particular interest are vaccinia and other pox viruses, as well as *Listeria monocytogenes*. These vaccines are difficult to use in a clinical setting owing to the formation of neutralizing antibodies. Protein- and peptide-based vaccines can be modified to increase their binding to MHC molecules, stimulating stronger T-cell responses. This has been performed with the peptide gp100 and the MART-1 analog peptide [92,93]. A major new advance was the introduction of Toll-like receptor ligands, which can activate APCs *in vivo* [94].

MelQbG10 is a vaccine that contains an immunogenic virus-like nanoparticle to which the antigenic peptide (melan-A/MART-1) is covalently coupled, as well as immunostimulatory oligonucleotides that trigger Toll-like receptor 9. The virus-like nanoparticle is a protein shell derived from the bacteriophage Q-β, which efficiently drains into local lymph nodes for uptake and processing by DCs and macrophages [95]. In a Phase II clinical trial, MelQbG10 was combined with additional adjuvants and/or the administration route was varied showing quantitatively and qualitatively different T-cell responses [96]. Prospective randomized clinical studies comparing vaccinated with unvaccinated patients are needed to determine whether the responses seen in Phase I and II testing are due to the vaccine or the natural biology of malignant melanoma.

Work on cancer immunotherapies for prostate cancer has proceeded furthest, in that the 2010 FDA approval of sipuleucel-T (Provenge®, Dendreon, WA, USA) has led the way for other similar approaches involving autologous vaccination regimens [2,97]. In this procedure, the peripheral blood mononuclear cells are collected from castration-resistant prostate cancer patients and cultured with a prostate antigen (i.e., prostatic acid phosphatase) and then coupled to GM-CSF. Patients are then given three infusions over several weeks. Although the approval of this treatment was seen as a great success for cancer immunotherapy, its high cost (about US$93,000 for the full course) has diminished its widespread use.

Adoptive T cell therapy

Over the last few years, there has been great focus on personalized medicine, although recent critics argue that a tumor is too heterogeneous and, therefore, a combination of 'mass' medication is probably more effective. However, personalized medicine in melanoma has demonstrated some success; melanoma patients who received lymphodepleting therapy and are subsequently transfused with self-derived and *ex vivo* expanded tumor-reactive lymphocytes have response rates of approximately 50% [98].

Adoptive T-cell therapy was developed by the Rosenberg lab in 1988 [99] and currently there are three strategies:

- TILs are obtained from a melanoma biopsy and amplified *ex vivo*. The lymphocytes arethen transfused back into the patient;
- Lymphocytes are obtained from the patient's peripheral blood and modified so that they express a chimeric antigen receptor (CAR). They are then amplified *ex vivo* and transfused back into the patient. A CAR is composed of an extracellular single chain antibody and an intracellular TCR signaling domain [100];
- Lymphocytes are obtained from the patient's peripheral blood and modified so that they express a recombinant TCR, which recognizes a melanoma-associated antigen. They are then amplified *ex vivo* and transfused back into the patient.

In all strategies, the patient is lymphodepleted in order to gain a stronger clinical response [101,102]. The different strategies all have advantages and disadvantages. Strategy one has less risk for autoimmunity, as in a recent study it was found that most TILs are not directed against known tumor antigens, but in fact are directed to unknown antigens, probably mutated self-proteins that are not expressed in other tissues [103]. In the last 10 years, TIL therapy has shown impressive results and according to the National Cancer Institute group, which summarized its 10 year experience with Phase II clinical trials, the clinical response rate is 51% and continuing complete regression over 5 years is 13% [104,105]. However, a disadvantage is that in a large number of cases it is impossible to isolate enough TILs from a melanoma sample. Furthermore, as this

technique is individualized and, therefore, time-consuming and expensive, it prevents its wider use. Recent developments to make the technique applicable on a larger scale include strategies two and three. Strategy two is independent from the functioning of the antigen-presenting machinery in melanoma. As a tumor-escape mechanism, melanomas often downregulate the expression of MHC molecules [106] while also deregulating the antigen-presenting machinery, which makes them unrecognizable by T cells. By using a CAR, the antigen is still recognized by the antibody and via the intracellular TCR signaling domain the T cell becomes activated. The method is still preclinical for melanoma; however, clinical trials in hematologic cancers show promising results [107,108].

The rationale for the third strategy is the possibility to acquire sufficient amounts of tumor antigen-specific T cells, which is often not possible with TIL therapy. However, the technique has some disadvantages, such as that the TCR is specific for one antigen presented on a specific MHC molecule. This means that immunoselection occurs where tumor cells that do not express the antigen will not be recognized. Furthermore, to date, only the MHC molecule HLA-A0201 is targeted and only patients who express at least one of these HLA molecules can benefit from the therapy, which is about 35% of the patients. There are also concerns about autoimmunity, as many tumor antigens are also expressed in normal tissue. In the last clinical trial, which had a clinical response rate of 30%, many patients experienced autoimmune effects [109]. Currently, further clinical trials are being developed and conducted to determine the potential for adoptive T-cell therapy in general melanoma care.

Anti PD1 therapy

The inhibition of PD-1 targets a different immunoregulatory mechanism as compared with other immune-activating therapies, in that its function focuses on the effector phase instead of the activation phase of the immune response (Figure 1). PD-1 is an inhibitory receptor on the T cell that, upon interaction with PD-1 ligand on the tumor cells, prevents the T cell from becoming active. Tumor cells can overexpress PD-1 ligand to escape immunological surveillance. Normal tissues, except for cells of the macrophage lineage, express little to no PD-1 ligand on their surface [110]. This tissue-specific expression suggests that abrogating the interaction of PD-1 with PD-1 ligand will have fewer side effects in the form of autoimmunity than, for example, blocking CTLA-4, which has autoimmune toxic effects in 20–30% of patients [111]. Inhibiting the interaction between PD-1 and PD-1 ligand can be achieved by using either anti-PD-1 antibodies or anti-PD-1 ligand antibodies. Both antibodies have been tested in Phase I trials; anti-PD-1 antibodies had an objective response rate of 41% with the optimal dose in melanoma, with 6% of all the treated patients (also patients with other tumors than melanoma) experiencing grade 3 or 4 drug-related adverse events [49], while anti-PD1-ligand 1 antibodies had an objective response rate of 29% with the optimal dose in melanoma, with 5% of the treated melanoma patients experiencing grade 3 or 4 drug-related side effects [48]. Phase II trials with anti-PD-1 antibodies will soon be started [206,207] and Phase III trials are planned.

As PD-1–PD-1 ligand interaction is playing a role in the effector phase of the immune response, combining anti-PD-1 therapy with a therapy that has its effect in the activation phase could be promising. Anti-CTLA-4 antibodies, which act in the effector phase, were the first immunotherapeutics to show a clinically important and statistically significant impact on patient survival [112]. Clinical trials combining anti-PD-1 and anti-CTLA-4 therapy are underway.

Furthermore, combining anti-PD-1 with a cancer vaccine that also works in the activation phase may provide some benefit. Cancer vaccines have been shown to induce the production of systemic antitumor-specific T cells [93,113,114]; however, these T cells are mostly ineffective in the clinic, since the immunologic inhibition in the tumor environment seems to be too strong. Overcoming this by simultaneously inhibiting PD-1–PD-1 ligand interaction in mice showed a tumor-eliminating response [115].

In another study using mice, combining anti-PD-1 with a therapy that stimulated DC maturation through activation of Toll-like receptor pathways (i.e., CpG therapy) showed a synergistic effect [116]. The same holds true for GM-CSF-secreting tumor-cell immunotherapy, which is based on the intradermal injection of tumor cells that are genetically modified to secrete GM-CSF [117]. Local GM-CSF secretion activates DCs, which will be targeted against tumor-associated antigens that are also present in the injected tumor cells. Both CpG and GM-CSF therapies work in the activation phase of the immune response.

Patient stratification criteria

Given the large number of uncharacterized genetic, epigenetic and probably environmental variables, not all patients respond to a given immunotherapy in the same way. For example, only 30% of the patients receiving CTLA-4-antibodies show a response [74]. However, almost all patients experience some side effects and stratification criteria such as better biomarkers are therefore needed to predict which patients will benefit from any particular treatment. To this end, most of the current research in the field is concentrated on the expression of certain immunologic factors that have an effect on tumor immunotherapy. These predictive markers could be the targets themselves; for example, PD-1 ligand. In a preliminary study, Topalian and coworkers found that 36% of the patients that were positive for PD-1 ligand expression had an objective response to anti-PD-1 therapy compared with none of the 17 patients with PD-1 negative tumors [49].

Other predictive biomarkers, aside from the molecular target itself, may be proteins that have an important role in either tumor progression or immune response; for example, the expression of MHC class I molecules. Tumor cells can evade immune recognition and elimination through the downregulation of MHC class I molecules on the tumor cells [118,119]. By downregulation of MHC class I molecules on tumor cells, they will be less recognizable to T cells. When the difference in MHC class I expression between regressing and progressing metastases from patients receiving immunotherapy was compared, only the progressing metastases downregulate MHC class I expression [120]. Furthermore, in another study where the HLA types in patients treated with IFN-α and IL-2 were checked, certain HLA types corresponded with a better response to treatment [121].

In addition to these immunologic factors, the predictive value of more general physiological markers have also been investigated, such as normal lactate dehydrogenase levels, a good performance status (e.g., Eastern Cooperative Oncology Group 0 or 1) [56], metastasis to less than three organs and cutaneous and/or subcutaneous metastasis [122], which were all associated with a better response to IL-2 therapy.

Future perspective: technology development

The difficulty in successfully using immunotherapy to cure cancer, despite intensive attempts, may be the result of an overly simplistic understanding of how the immune system functions. More intelligent combination therapies are needed that influence multiple stages of the immune reaction to elicit a synergistic effect. For example, vaccination with mRNA-encoding tumor antigens is a new strategy where antigen presentation and immune stimulation are combined [123]. In contrast to normal RNA, which is degraded in a few minutes and cannot act as an immune stimulus, stabilized RNA can act as pathogen-associated molecular patterns, which are recognized by the immune system [124]. On the other hand, stabilized RNA is hard to translate by a cell, which is required for antigen presentation. Recently, a new vaccine combining stabilized and unstabilized RNA has been developed with promising preclinical results [98,123,125,126].

It is likely that in the future, more basic research on the immune system and tumor immune evasion mechanisms may reveal additional targets for pharmaceutical or biologic intervention through better antigen identification and targeted immunoregulation. For instance, in addition to the demonstrated success of CTLA-4 and PD-1, blocking TIM-3 and LAG-3 (i.e., two additional inhibitory receptors expressed on T cells) shows promising results. A recent study in mice with solid tumors showed that combining Tim-3 blockade with PD-1 blockade is more effective than blocking either receptor alone [127]. Another study suggested that LAG-3 and PD-1 also work synergistically [128].

Finally, the way drugs are delivered is likely to influence their effectiveness. With localized therapy, higher doses can be reached and combinations that previously were not possible owing to systemic side effects become feasible. The recent example of nanolipogels demonstrates this well. These very small (i.e., nanoscale), hollow and biodegradable spheres are able to carry the drugs through the bloodstream to the tumors, where they ultimately get trapped in the tumor vasculature [3] and release their drugs. In a recent study, nanolipogels with a TGF-β-inhibitor and IL-2 were injected into mice with melanoma [3]. The results showed that the tumors went into remission, their growth was delayed and the survival of the mice was prolonged. By combining these two drugs, the mouse immune response was boosted with IL-2 and the secretion of tumor defense molecules was inhibited with anti-TGF-β. This combination treatment had not been possible previously owing to systemic side effects [3]. However, with this technique, the combination therapy was localized and side

effects were greatly reduced. A Phase I/II clinical trial analyzing the safety, tolerability and OS combining the specific BRAF kinase inhibitor vemurafenib with ipilimumab in BRAF-mutated melanoma patients is ongoing [208].

These and other combinatorial approaches may start a new wave of therapeutic strategies that use immunoregulatory agents to target different aspects of tumor growth, metastasis and immune evasion. From the dramatic increase in interest regarding cancer immunotherapy and many recent successes, it is clear that immunotherapy is back on the list of antimelanoma therapies and likely to yield greater results in cancer patients as our understanding of the immune system and tumor-evasion mechanisms improves in the coming years.

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Executive summary

- Cancer immunotherapy has a long history, and recent successful application suggests a promising future in various cancer types.
- Past failures in cancer immunotherapy such as the CD28 superagonist are a cautionary tale in immunomodulatory treatment.
- The administration of the CD28 superagonist in healthy human volunteers elicited a massive cytokine storm within 12–16 h postadministration.
- An alternative to direct modulation of the immune system is the identification of tumor-specific antigens that could be used for immune therapy. Cancer testes antigens (CT-antigens) and melanocytic differentiation antigens are examples of such targets.
- Current cancer immunotherapy for melanoma consists of regimens involving IL-2, interferon, targeting of the inhibitory receptor CTLA-4, or inhibiting the interaction of PD-1 ligand with its receptor.
- IL-2 therapy has shown little improvement in overall patient survival; although interferon was shown to improve relapse-free and overall survival, it is also associated with an extensive set of side effects; CTLA-4 targeting (ipilimumab) has resulted in a significant increase inmedian overall survival.
- Melanoma vaccines, although showing great promise, have so far failed to demonstrate a significant therapeutic effect.
- Adoptive T-cell therapy, although quite complex, has shown strong clinical response rates. But this treatment is both cost-prohibitive and time-consuming, thus limiting its general applicability.
- Treatment of the effector phase of the immune response, through PD-1 inhibition, has resulted in high objective response rates in clinical trials and shows great promise for more general use.
- The identification of patient stratification criteria for melanoma immunotherapy will be critical to determining which patients might benefit from any particular treatment as well as for designing potential combination therapies.

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10. Review 2: Metastatic melanoma moves on: translational science in the era of personalized medicine

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Metastatic melanoma moves on: translational science in the era of personalized medicine

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Abstract Progress in understanding and treating metastatic melanoma is the result of decades of basic and translational research as well as the development of better *in vitro* tools for modeling the disease. Here, we review the latest therapeutic options for metastatic melanoma and the known genetic and non-genetic mechanisms of resistance to these therapies, as well as the *in vitro* toolbox that has provided the greatest insights into melanoma progression. These include next-generation sequencing technologies and more complex 2D and 3D cell culture models to functionally test the data generated by genomics approaches. The combination of hypothesis generating and hypothesis testing paradigms reviewed here will be the foundation for the next phase of metastatic melanoma therapies in the coming years.

Keywords Melanoma · 3D culture · Translational research · Bioengineered skin · Skin reconstructs · Melanoma treatment

Great discoveries often lag far behind technological innovations; real-world, transformative applications are even slower to materialize. It took 100–150 years to adapt the ʙreading stone^ lenses of the twelfth century into spectacles to correct hyperopia and myopia, and another two centuries to build telescopes out of lenses that allowed Galileo to support the Copernican model of a heliocentric solar system.

Although the pace of translational science has greatly accelerated since the Middle Ages, the journey from theory, discovery, and application to measurable clinical benefit has been slow for most cancer therapies. This has been especially true for metastatic melanoma, which has only recently benefited from decades of basic research. However, within the last 10 years, metastatic melanoma has gone from being almost untreatable to one of the most promising examples of evidence-based, personalized medicine today. It was not until the publication of some landmark papers in 1996 and 2002 that the foundation was set for two of the most disruptive technologies in the treatment of metastatic melanoma: targeted therapy and checkpoint inhibition [1–3]. These discoveries themselves were the results of decades of technological advances and basic research in next-generation sequencing technologies and immune biology, to name a few [4, 5].

While the recent successes of single-agent and combination therapies have been encouraging, there is still a great deal of work to be done to better stratify patients for the appropriate treatment, to understand and circumvent therapeutic resistance, and to identify new targets for therapy. Below, we review the latest technological developments in these areas, the scientific results that will inform the next generation of therapies, and the challenges we still face in translating basic discoveries to treatments that will ultimately benefit patients. The selection of papers is focused on the technologies that have yielded the most clinically relevant results in recent years regarding metastatic melanoma progression: next-generation sequencing tools and *in vitro* functional assays.

1 Melanoma treatment

The majority of malignant melanoma cases are detected at an early stage where surgical excision is curative. However, once melanoma has spread to a distant site, disease control becomes difficult. After decades of disappointing clinical studies in metastatic melanoma patients, the last 10 years have shown some

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exciting successes with clear clinical benefit. The current treatment paradigm for our metastatic melanoma patients follows the recently published treatment guidelines (Fig. 1) [6].

Early studies in B-rapidly accelerated fibrosarcoma (BRAF)-mutated patients investigated small molecules that blocked a range of kinases including RAF (sorafenib, RAF265), but more potent and selective inhibitors that targeted mutated BRAF (particularly at amino acid 600) were more recently tested. These revolutionized the treatment of BRAF-mutant melanoma [7–11]. Phase III trials of both vemurafenib and dabrafenib demonstrated superiority over chemotherapy, as did the mitogen-activated protein kinase kinase (MEK1/2) inhibitor trametinib. However, within 5–7 months, disease progression was observed with single-agent BRAF and MEK inhibitors [12–14]. In three randomized trials (COMBI-d, COMBI-v, co-BRIM), combined targeting of BRAF/MEK (i.e., encorafenib and binimetinib, dabrafenib and trametinib, or vemurafenib and cobimetinib) also showed improved response rates, progression-free survival, and overall survival than either therapy alone [15–20]. But resistance (innate or primary, and acquired or secondary) is still a major challenge in the targeted-therapy paradigm.

To date, immunotherapy with high-dose IL2 or checkpoint inhibitor antibodies against cytotoxic T-lymphocyte associated protein (CTLA-4) and/or programmed cell death protein (PD-1), and targeted therapies (BRAF and MEK inhibitors) in patients with a BRAF V600 mutation. Also in NRAS mutated patients (the MEK inhibitor), have shown clinical benefit [6]. The use of BRAF inhibitors has significantly improved overall and progression-free survival in BRAF V600-mutated patients [21]. Unfortunately, most patients develop resistance after some months.

Several theoretical models have been developed to understand how resistance may arise. These include a subclonal evolution model, phenotype switching, or cancer stem cells [5]. Like most models, each of these has some relevance and strong supportive data, but also major caveats. Understanding this complexity outside of the dogma associated with any particular model will facilitate a more accurate representation of how therapeutic resistance exists in a real-world clinical setting.

1.1 Genetic mechanisms of therapeutic resistance (subclonal evolution)

There is some evidence that resistance may follow aspects of a Darwinistic evolution model in which selection and adaptation play vital roles. Small populations of resistant melanoma cells might already exist before treatment, either by genetic or epigenetic means, which are then selected during treatment due to the advantage they have over non-resistant cells [22]. Resistance could also develop during the course of treatment when the cells adapt to the new stressor. This too could happen via alterations in the genotype or epigenome resulting in phenotypic changes.

Immunohistochemical analysis of post-resistant samples fromthephase IIBRIM-2 vemurafenibtrialrevealedthatnearly all resistant samples had a reactivation of the MAPK pathway [23]. Since then, several genetic as well as non-genetic adaptive mechanisms for MAPK therapy have been described. A meticulousanalysisofpre-treatment, on-treatment, andpost-resistant biopsies has shown elevated pERK1/2 levels in progressive tumors and gain-of-function mutations in MEK and NRAS that reactivate MAPK signaling [24].

The first paper describing this reactivation demonstrated a *de novo MEK1* mutation (P124L) in a treatment-resistant metastasis from a BRAF-mutated patient who was successfully treated with a MEK inhibitor (selumetinib) [25]. This mutation conferred substantial resistance to MEK inhibitor therapy and only moderate resistance to a BRAF inhibitor. MEK mutations can also occasionally confer resistance to both BRAF and MEK inhibitors [26]. More commonly, concomitant NRAS mutations have been observed with persistent BRAF mutation [26–29]. This is especially interesting because of the paradoxical activation caused by BRAF inhibitors, which has been demonstrated for cutaneous epithelial malignancies [30, 31]. It may be that the presence of an NRAS mutation under BRAF inhibitor therapy may result in a detrimental effect because of this paradoxical activation.

In a large sequencing study that is described further in the genomics section below, Shi et al. analyzed the genomes of 100 tumor samples derived from 44 patients, which consisted of 29 pre-treatment samples and 71 resistant post-treatment samples [28]. All post-treatment samples still contained the $BRAF^{V600E/K}$ mutations, indicating that non-mutated subclones were not being selected during treatment. In line with the BRIM-2 analysis, the most common resistance mechanism they identified was MAPK reactivation (in 70% of the cases), either by an additional NRAS or KRAS mutation, mutant BRAF amplification or BRAF alternative splicing, or by CDKN2A loss. BRAF amplifications and truncated BRAF variants have also been reported elsewhere [32]. BRAF splicing variants result in a truncated BRAF protein, which lacks a RAS-binding domain, thereby conferring BRAF inhibitor resistance through dimerization and pathway activation [33].

They also identified the PI3K-PTEN-AKT pathway as a second important resistance pathway (22% of their post-treatment samples contained mutations in PI3K-AKT regulatory genes). By *in vitro* overexpression and knockdown studies, they could show that melanoma cells overexpressing $AKT1^{Q79K}$, $AKT1^{E17K}$, $AKT3^{E17K}$, $PIK3CA^{D350G}$, $PIK3CA^{E545G}$, $PIK3CA^{E545K}$, or $PIK3R2^{N561D}$ were more resistant to BRAF inhibition compared to cells overexpressing the WT variant of AKT1, AKT3, PIK3CA, and PIK3R2, respectively. In addition, PTEN knockdown in a PTEN WT cell line introduced vemurafenib resistance. When they analyzed

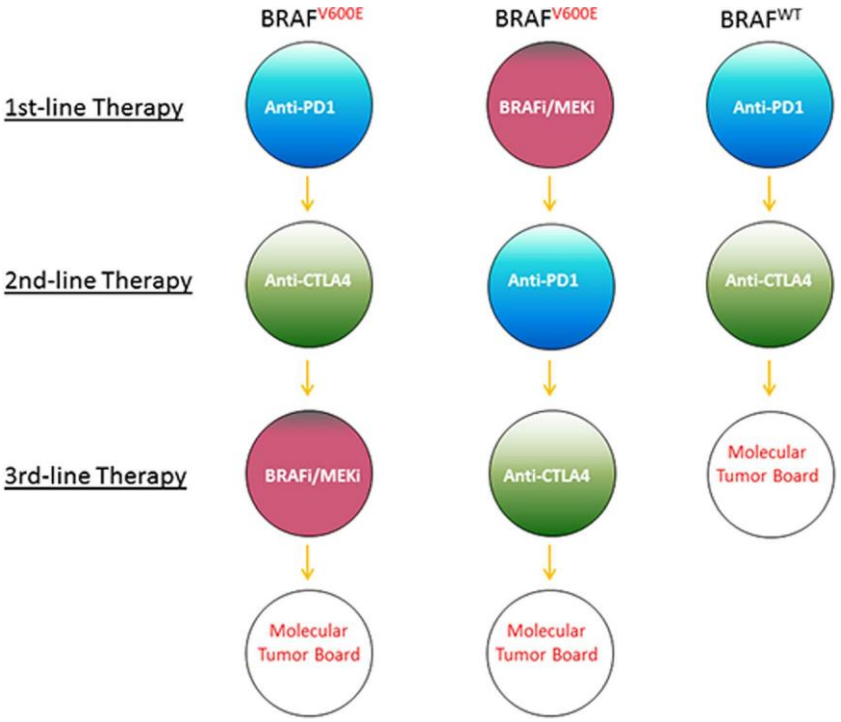

Fig. 1 Current treatment guidelines for metastatic melanoma [6]

nine different resistant post-treatment samples from one patient, they identified at least five different resistance mechanisms, indicating that a single biopsy from one sample is not sufficient to determine follow-up inhibitor treatment after BRAF inhibitor resistance.

1.2 Non-genetic mechanisms of therapeutic resistance (phenotype switching)

Disease progression occurs in most patients after successful tumor control with mono- or combination therapy for months or even years. The response duration is generally quite broad (i.e., from weeks to years), and the causal mutations driving treatment failure are described above. In the context of the phenotype switching model, the transcriptional response to MAPK pathway inhibition can be considered to be proliferative cells activating mesenchymal (i.e., invasive or stem-like) expression modules that are associated with reduced glucose metabolism [34]. The melanoma cells may thereby buy time to activate alternative *signaling* pathways and enable subclones with additional activating mutations to flourish in the new microenvironment.

Besides MAPK pathway reactivation, activation of parallel signalling pathways such as the phosphoinoside-3-kinase (PI3K) pathway can also occur, for instance through the activation of the kinase COT [35–37]. This can occur, for instance, through the activation of the kinase COT [38]. The tumor stroma can also play an important role in bypass *signaling*. Stroma cells from resistant tumors can secrete growth and viability factors that protect melanoma cells from BRAF inhibition. These include the hepatocyte growth factor (HGF), which can facilitate tumor cell growth through the paracrine activation of the HGF receptor MET, which leads to PI3K pathway activation [39, 40]. Other receptor-tyrosine kinases (RTKs) may enable proliferation in the presence of a BRAF inhibitor. While melanoma cells can express the epidermal growth factor receptor (EGFR), the levels are quite low compared to colon or squamous cell carcinomas. However, upon innate or acquired resistance, melanoma cells can upregulate the autocrine secretion of EGF and EGFR [41, 42]. This suggests that, indeed, the tumor stroma is an active participant in the response to targeted therapies and may play a crucial role in the attenuation of therapeutic success.

Other avenues for intervention strategies may arise from a more complete understanding of the biochemical and metabolic events that are associated with MAPK reactivation, such as the maintenance of eIF4F complexes and the persistent ERK-independent phosphorylation of 4EBP1 or the enrichment of pro-apoptotic BCL-2-modifying factor-dependent degradation of eIF4G [43]. Interestingly, interferon beta secretion was also observed to occur with tumor regression in the presence of BRAF inhibitor therapy. In addition, PKCε-phosphorylated ATF2 downregulates IFNβ1, which elicits therapeutic resistance (Lau et al. Oncogene in press).

Genetic mutations may confer permanent drug resistance. However, it has been observed that before the drug resistant stage, an adaptive and transient stage of reversible drug resistance exists. Upon stress, such as inhibitor treatment,

melanoma cells undergo an innate response which allows them to better tolerate drugs [44]. This response is characterized by de-differentiation (reduced expression of melanocytic lineage-specific markers), increased expression of stemness and EMT markers, and increased expression of genes involved in epigenetic remodeling. Of the latter group, the enzyme KDM5B is a marker for a slow-cycling phenotype with increased drug tolerance [45, 46]. This enzyme was upregulated in the stress response, as well as other markers, pointing to a downregulation of transcription and replication pathways, suggesting a slow cycling semi-quiescent state. In this stationary phase, cells change morphology to a flattened and enlarged phenotype and increased senescence associated SA-β-Gal activity, H3K9me3-positive heterochromatic foci, and promyelocytic bodies (PML) bodies [47]. Other stressors, such as hypoxia or low glucose, can induce a similar stress response [48]. Interestingly, when these stressed hypoxic or low glucose cells are exposed to various drugs, they are more resistant than their non-stressed equivalents.

In the previously mentioned study from Shi et al., the authors further investigated the genomes from 13 tumor samples derived from 1 patient [28]. Two samples were prior to treatment, two samples were taken during treatment, and nine samples were disease-progressive tumors taken after treatment. The tumors taken during treatment expressed little to no Ki-67 staining in immunohistochemistry compared to pre-treatment samples, indicating a senescent-like state with no proliferation. To the contrary, the post-treatment samples showed the highest level of Ki-67 staining; moreover, the two tumors from the post-treatment samples with the most extensive genetic divergence displayed the highest proliferative activity, suggesting increased tumor fitness with decreasing C > T transitions, suggesting the presence of non-UV-related mutagenic processes upon progression.

Early adaptive signaling upon BRAF inhibitor therapy can occur as soon as 24 h after initiation of therapy [49]. Responses described include activation of the PI3K pathway [50], an altered oxidative metabolism [51], and upregulation of ERBB3 expression [52]. This rapid response, as well as the observation that melanoma primary tumors and metastases are very heterogeneous at multiple levels (i.e., genetic, epigenetic, transcriptional, proteomic, metabolomic, etc.), suggests that subclonal evolution or the stem cell model does not play much of a role in the acute phase of treatment response. This observation and some compelling publications over the last few years have generated a widely accepted opinion in the melanoma community that these models alone fail to adequately account for heterogeneity and the early phases of resistance [5]. These subpopulations can contribute to tumor progression by exhibiting expression-driven, treatment-resistant slow-cycling behavior (such as JARID1B expressing cells) and tumor cells with features of epithelial to mesenchymal transition or mesenchymal phenotypes and expression patterns. This plasticity may enable a dynamic response to external stressors such as therapies and may further generate a non-hierarchical organization of the tumor, which allows for a more rapid adaptation to the sudden reduction of MAPK signaling [53]. Interestingly, the limited cell death observed *in vitro* and *in vivo* during targeted therapy may be the result of proliferative tumor cells switching their epigenetic and transcriptional states to allow for a more slow-cycling mesenchymal phenotype that can better tolerate the therapeutic intervention. Resistance to BRAF inhibitors has also been associated with high, pre-treatment expression of anti-apoptotic BCL2 proteins (i.e., BCL2 and BCL2A1) [54, 55]. This may explain the limited apoptosis that has been observed upon BRAFi treatment.

The role of MITF in resistance is conflicting, as roughly 50% of resistant tumors have an increased expression of MITF, whereas the other 50% show a decreased expression as an early response to BRAF inhibitors. Even within a patient, different relapsing tumors can have enhanced or absent MITF expression [56]. An interesting observation is that in (vemurafenib) resistant cell lines, the presence of MITF indicated resistance to BRAF inhibition but retained responsiveness to MEK or ERK inhibition, whereas absence of MITF was observed in cells that were also resistant to other MAPK pathway inhibitors [56]. The authors also noted that cells without MITF expression can better tolerate higher drug doses. In addition, when they checked for intrinsic resistance in treatment-naive melanoma cell lines, they found that if a BRAF- or NRAS-mutated melanoma cell line was intrinsically resistant to BRAF or MEK inhibitors, it had low MITF expression, which is probably due to the higher expression of RTKs such as AXL, EGFR, and PDGFRb in MITF-low cells, as the inhibition of those receptors, especially AXL, sensitized the cell lines to MAPK inhibitors [56].

In cases of BRAF inhibitor resistant cells with elevated MITF expression, Nelfinavir (an HIV1-protease inhibitor) might be an interesting therapy [57]. Inhibition of BRAF or MEK reduces the expression of SKI, which leads to an upregulation of PAX3, and this in turn increases the expression of MITF [57]. In mice, combination treatment with Nelfinavir prevented the MAPK inhibitor-induced upregulation of PAX3 and MITF levels, thereby sensitizing the melanoma cells to MAPK pathway inhibition. However, whether elevated MITF expression is a direct cause of resistance has to be further evaluated.

2 Non-cell autonomous resistance

Interestingly, the classical theoretical models of melanoma progression tend to be cell-autonomous and do not leave much of a role for the interplay of external factors such as the tumor microenvironment. Darwin also postulated that species that learn to collaborate have a better chance to prevail. In a non-

treated tumor, sensitive cells will generally have a growth advantage over resistant cells. However, upon treatment, resistant cells will have the upper hand, and it might even be so that sensitive cells support the resistant cells in this situation [58]. Upon treatment, sensitive cells secrete a therapy induced secretome (TIS), which was found to contain many mediators directly or indirectly activating the AKT pathway in resistant cells, who therefore became more proliferative upon treatment [58]. Treating mice with a combination of MAPK pathway and AKT pathway inhibitors could prevent this TIS-induced accelerated expansion of resistant cells in tumors [58]. Resistant cells in turn can help sensitive cells by preventing dendritic cell maturation and activation. When treated with BRAF inhibitors, sensitive melanoma cells express danger signals that incite an immune response, and the presence of resistant cells has a dampening effect on that response [59].

Collaboration can also take place with other cells in the tumor microenvironment. In a sophisticated *in vivo* mouse model where intravital imaging was combined with a biosensor construct that could monitor ERK/MAPK activity in live tissue, Hirata et al. observed that melanoma cells respond to BRAF inhibition by reducing pERK; however, in tumor areas with high stromal density, cells rapidly recovered pERK expression to normal levels as soon as 1 day after the initial dose [60]. In mice, these tumors were resistant; however, when cultured as pure melanoma cultures *in vitro*, the cells were sensitive to BRAF inhibition, suggesting that the microenvironment aids melanoma cells to withstand inhibitor treatment. Indeed, when they co-cultured the melanoma cells with the tumor-associated fibroblasts, the melanoma cells conferred resistance to BRAF inhibition. It appeared that fibroblasts, upon treatment with BRAF inhibitors, paradoxically activate the MAPK pathway via PDGFRa upregulation which let them remodel the extracellular matrix in a denser collagen fibril matrix. This stiff matrix has changes in integrin organization and FAK signaling and directly provides a safe haven for melanoma cells. Adding FAK inhibitors to the BRAF inhibitor resensitized the melanoma cells to BRAF inhibition [60].

Lastly, next to tumor cell mutations and adaptations and microenvironmental factors, patient factors possibly also play an important role in resistance. How well the drug is absorbed in the intestine (e.g., receptors on intestinal cells, intestinal flora drug metabolism), how much of the drug is cleared in the liver (enzyme activity), how much of the drug is bound to albumin (starvation/feeding status), and how well the drug can reach the tumor (vasculature, location of the tumor) all may have an influence on the drug concentration that effectively reaches the tumor cells. However, as every patient is unique, this is very complicated to investigate in an evidence-based medicine approach.

One of the factors that can be investigated and maybe compensated, however, is age. In a small cohort of 79 patients, those younger than 65 years had almost a double reduction in tumor burden upon MAPK inhibitor therapy than older patients due to more resistance in the latter [61].

The authors found that sFRP2 levels are increased in the serum of patients older than 55 years compared to younger patients, probably due to an increased secretion by aged senescent fibroblasts. This increased sFRP2 expression caused a reduction in β-catenin, MITF, and APE1 expression. A loss of APE1 leads to more cellular damage by ROS. Indeed, melanoma cells exposed to aged fibroblasts or aged fibroblast conditioned medium *in vitro* have greater DNA damage. This damage could be reversed by inhibiting ROS activity. Decreases in β-catenin and MITF and increases in ROS have been linked to BRAF inhibitor resistance. The authors could show that *in vitro* and *in vivo* melanoma cells treated with conditioned medium from young fibroblasts or implanted in young mice were more sensitive to BRAF inhibition than those treated with the conditioned medium from old fibroblasts or implanted in old mice, respectively.

3 Melanoma genomics

Undoubtedly, one of the major transformative technologies driving translational research today is next-generation sequencing (NGS). Although genetics has been known to contribute to melanoma progression for decades, the first pivotal study identifying BRAF as the most important driver gene revealed that melanoma is a genetic disease and highlighted the importance of the MAPK pathway [2]. It was the discovery of BRAF V600 that was mutated in 50% of the melanoma samples with V600E comprising 80% of the mutations. Another observation from this study was that NRAS mutations, mutated in 15% of the melanoma samples, were mutually exclusive to BRAF mutations [2]. The scope of this study was just focused on the MAPK pathway (i.e., RAS-RAF-MEK-ERK), but revealed crucial information about the genetics of melanoma. Many subsequent NGS studies used whole exome (WES) or whole genome sequencing (WGS) on melanoma tumors, melanoma short-term cultures, or melanoma cell lines. In this section, we will focus on the large cohort studies for a comprehensive review of genomic analyses in melanoma [62].

The first genomic characterization of melanoma was a WGS study performed on the melanoma cell line COLO-829 [63]. An ultra-violet radiation (UVR) signature, defined by C > T substitutions [64], was detected in this cell line. This finding supported the notion that UVR is an environmental risk factor for melanoma. Two of the largest (WES) studies looking at 121 melanoma tumors and 147 melanoma tumors confirmed the UVR signature for sun-exposed melanomas and uncovered that non-sun-exposed melanomas exhibited a lower mutation rate and different mutation signature, thus highlighting the differences in melanoma subtypes, such as acral, mucosal, and uveal melanomas [65, 66]. These two studies together detected known melanoma oncogenes such as BRAF and NRAS and in addition uncovered

new oncogenes like RAC1, PPP6C, and STK19 and new tumor suppressors like ARID2, DCC, TACC1, SNX31, NF1, ZNF560, FAM58A, and ME1. Copy-number analysis revealed losses in known tumor suppressors like PTEN and CDKN2A and gains in known melanoma oncogenes like MITF, CCDN1, CDK4, and TERT. These two studies provided a great insight into the genomic landscape of melanoma and also highlighted the fact that driver mutations in BRAF and NRAS do not likely occur from UVR. This suggests that the initial oncogenic transformation is UVindependent. Exome sequencing of $BRAF^{V600E}$ mice treated with UVR revealed a typical UVR mutation signature across the exome and inactivating mutations in *Trp53* [67], thus demonstrating the direct link of UVR to melanomagenesis.

WGS of 25 metastatic melanomas revealed frequent PREX2 mutations and TERT promoter mutations [68, 69]. WGS also confirmed the UVR signature and as well revealed many structural rearrangements in the genome of known oncogenes like ETV1. TERT promoter mutations were also seen in an independent study looking at families with a history of melanoma and were also validated in sporadic melanoma samples [70]. WES has also been used for phylogenetic analysis of metastatic progression of melanoma [71, 72]. Each study had eight patients where the primary tumor and multiple metastases were subjected to WES. Both studies found that metastasis formation is not a simple linear progression of mutation accumulation, but exhibits a quite complex evolution from the primary tumor. The metastases can be founded from multiple subgroups of the primary lesion. These findings have great implications for therapy as inter-tumoral and intra-tumoral heterogeneity can play a large role in response to treatment.

Thenextlargestomicsstudycamefrom The Cancer Genome Atlas melanoma working group [73]. In this study, 67 primary tumors and 266 metastatic tumors were analyzed by WES, RNAseq, copy number, and reverse phase protein array. Two new molecular subtypes were defined in this study. Tumors that were both BRAF and NRAS wild-type often had a mutation in NF1 (45%). Since no high-frequency hotspot mutations occurredin NF1, itwas assumed that NF1 was a tumor suppressor. NF1 is known as a negative regulator of RAS, thus loss of function mutations would lead to activation of the MAPK pathway. Tumors that were triple wild-type for BRAF, NRAS, and NF1 weretypicallylesslikelytohavea UVRsignatureandwere morelikelytohavecopy-numberalterationsandcomplexstructural rearrangements. Interestingly, there were no significant mutations associated with metastasis suggesting progression to metastasis may be patient specific.

Thenexttwo WESstudiesanalyzed 501 and 213 melanomas and confirmed the NF1 molecular subtype. Inaddition, RASA2 mutations were discovered to significantly co-occur with NF1 mutations [74, 75]. It was observed that the NF1-mutated melanomas had a higher mutation rate than BRAF or NRAS mutated melanomas suggesting that NF1 alone is not sufficient to drive melanomagenesis and additional mutations are needed such as mutations in RASopathy genes. RASopathies are developmental disorders caused by mutations in genes in the RAS/MAPK pathway. For instance, germline mutations in NF1 cause neurofibromatosis. Many melanoma mutations share known RASopathy gene mutations, reviewed in [76], suggesting that patients with a RASopathy could have a higher chance of developing melanoma.

One large WES study interrogated the mutation progression of melanocytic nevi to melanoma [77]. This study sequenced 37 FFPE melanomas with histologically distinct precursors. The authors sequenced by WES 150 distinct areas. Benign nevi generally had one mutation, which was usually BRAF V600E. Intermediate lesions had at least two pathogenic mutations and had a higher mutation rate than benign lesions. BRAF V600E mutations occurred in intermittent sun-damaged skin and in younger patients, whereas BRAF V600K or K601E and NRAS mutations occurred in chronic sun-damaged skin and older patients. TERT promoter mutations were found in a significant portion of the intermediate lesions. Once a melanoma becomes invasive, inactivation of CDKN2A occurs. TP53 and PTEN mutations were observed to occur in late stages of invasion. This study elegantly dissected the stepwise genetic evolution from melanocytic nevi to invasive melanoma.

4 Melanoma transcriptomics

One of the first studies to apply RNAseq to melanoma samples discovered 11 novel gene fusions and 12 novel chimeric transcripts and, in addition, validated 29 somatic mutations in 10 melanoma samples [78]. The study did not detect any of the gene fusions in an additional 90 samples, suggesting gene fusions are private events.

The TCGA has one of the largest collections of RNAseq data with 470 melanoma samples with supporting clinical data. From the TCGA melanoma landmark study, RNAseq analysis uncovered three signatures of gene expression across these melanoma tumors: a group with high immune gene expression, a group with high keratin gene expression, and a group with low MITF gene expression. Survival analysis of these three groups revealed the keratin group to have the lowest survival rate and the immune group to have the highest survival rate, suggesting biological significance to these gene signatures. BRAF hotspot mutations were more likely to be found in the MITF-low group (66%), and the MITF-low group generally contained samples with a mutation in BRAF or NRAS or NF1 compared to the keratin and immune group (3% vs. 21% and 14%, respectively).

Before NGS, microarrays were a popular tool to dissect the molecular characteristics of melanoma. Microarray analysis of 218 melanoma cell cultures across 6 different studies revealed two gene expression patterns, a вproliferative^ signature and an вinvasive^ signature [79]. The вproliferative^ signature

was defined by high expression of typical melanocytic genes like MITF, TYR, LEF1, and SOX10, and the ʙinvasive^ signature was defined by high expression of WNT5A, SOX9, TGFB, and TCF4. This signature was found to be independent of primary or metastatic lesions and of mutated BRAF V600E. These two signatures had phenotypic consequences *in vitro*, with the ʙproliferative^ signature melanoma cells having a fast doubling time and limited invasive capacity, whereas ʙinvasive^ signature melanoma cells had a slow doubling time and high invasive capacity, and the melanomas were able to switch back and forth between the two phenotypes [80]. This has been termed phenotype switching. A recent study demonstrated that the TCGA RNAseq dataset also contained these two signatures [81]. No significant mutations were associated with either phenotype, suggesting these two cell states are driven by transcriptional reprogramming and not by genetic events. We have recently shown that DNA methylation of the SOX9 promoter is at least one of the mechanisms that regulates this phenotype by the mutually exclusive developmental transcription factors SOX10 and SOX9 that could control aspects of each phenotypic state (Fig. 2) [82, 84].

Single-cell RNAseq has also dissected the heterogeneity of melanoma [85]. In this study, 4645 cells were isolated from 19 melanoma tumors and subjected to single-cell RNAseq. One interesting finding from the study was the MITF high signature and an AXL high signature which resembled the proliferative and invasive signatures described before. At the bulk tumor level, the tumor could be classified as MITF high or AXL high, but at the single cell level, a spectrum could be found. The tumor also contained cells with high cycling capacity and low cycling capacity. Along with the tumor cells, stromal cells and lymphocytes were also sequenced. Cancer-associated fibroblasts (CAFs) had a significant association with the AXL high signature and an anti-correlative association with the MITF high signature. CAFs and melanoma cells could both express an AXL signature suggesting tumor-stromal interactions where CAFs could influence the transcription profile of melanoma cells. CAFs expressing C3 also had a significant association with $CD8^+$ T cells. Briefly, the lymphocyte population could be distinguished by their specific identities and subsets by known gene markers and even identifying exhausted T cells. Exhausted T cells were linked to T-cell expansion, whereas non-exhausted T cells were not expanded. Overall, this study has provided great insight into the transcriptomic heterogeneity of melanoma and the interaction of melanoma cells with the environment of stromal and lymphocytic cells.

5 Discovery of resistance mechanisms to therapy by genomics and transcriptomics

NGS has also been a great boon for the study of drug resistance mechanisms in melanoma. One of the first studies using gene

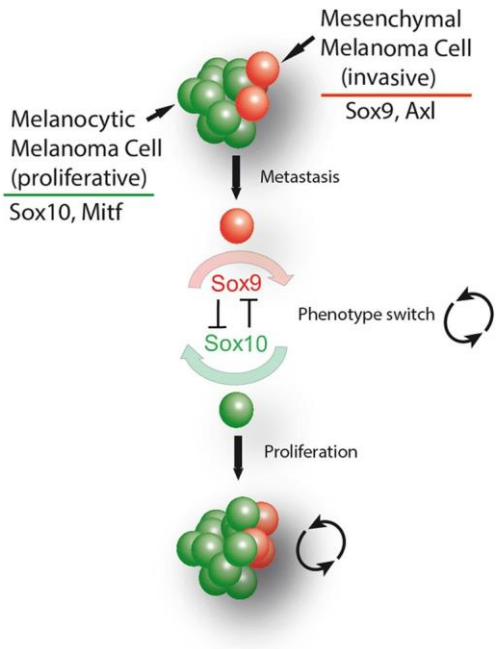

Fig. 2 Current model of melanoma heterogeneity (i.e., phenotype switching), whereby SOX9 and SOX10 represent mutually exclusive repressors of the alternative fate and switching of phenotypic states is driven by microenvironmental factors and epigenetic programming [5, 82, 83]

expression profiling discovered that upregulation of PDGFRB leads to resistance to BRAF V600E inhibition [27]. In that same study, mutations and upregulation of NRAS also conferred resistance to BRAF V600E inhibition. Targeted sequencing of 138 cancergenes uncovered a MEK1 mutation (C121S) to confer resistance to BRAF V600E inhibition [86]. WES of 20 patients uncovered $BRAF^{V600E}$ copy number gain [87]. WES of FFPE material from 45 patients revealed MEK2 mutations and some MEK1 mutations that confer resistance to vemurafenib [88]. MITF amplification was also detected in this study to confer resistance. Multiple resistance mechanisms were also seen to evolve in parallel within one tumor. One patient had two distinct NRAS mutations that were detected mutually exclusively on reads that spanned the same locus. The authors suggested that the two mutations occurred *in trans* in the same resistant tumor or represent separate subclonal resistant populations in the tumor.

Earlier, we described the WES treatment-resistance study of Shi et al. [28], which detected 22% of the resistant tumors had a perturbation in the PI3K-PTEN-AKT pathway, which included mutations in AKT1/3, PIK3CA, PIK3CG, PIK3R2, PTEN, and PHLPP1. The authors observed that 20% of the resistant tumors could have more than one resistance mechanism, suggesting tumor heterogeneity and/or collaborative mechanisms. Phylogenetic analysis of multiple resistant tumors from the same patient revealed branched evolution rather than linear evolution suggesting resistant mechanisms can evolve in parallel in different tumors. RNAseq analysis of

the same patient cohort revealed that transcriptional processes were highly consistent in the resistant tumors, which is in contrast to genetic events that were more heterogeneous [89]. cMET upregulation and LEF1 downregulation occurred in the majority of resistant tumor cells. These two genes were observed to have a strong anti-correlation with DNA methylation, suggesting an epigenetic mechanism to BRAF inhibitor resistance. In addition, upregulation of M2 macrophage markers could be seen in a subset of the tumors which was also associated with reduced expression of T-cell markers. The authors suggest that the involvement of the immune system is also an important component to BRAF inhibitor resistance. It is interesting to note the importance of LEF1. It is a gene part of the proliferative signature in the phenotype switching model. We have observed that cells with a proliferative phenotype are generally sensitive to BRAF inhibition and upon resistance the cells adopt an invasive signature, thus supporting the notion that downregulation of LEF1 leads to resistance [34]. In the single-cell RNAseq study by Tirosh, the authors also observed that cells adopt an invasive signature, AXL high MITF low, when resistance occurs [85]. These studies highlight the role that phenotype switching has in resistance to BRAF inhibitors (see Kemper et al. for a comprehensive review [90]). Again, based on our initial findings published in cancer research 2008 [80], these two profiles have been predicted and coined proliferative (MITF high) and invasive (MITF low AXL positive) phenotypes [91]. Resistant tumors are characterized by a strong expression of gene sets that we have called invasive gene sets representing mesenchymal transition, cell adhesion, extracellular matrix remodeling, angiogenesis, and stemness [80].

Immunotherapy with anti-PD-1 antibodies is a preferred first-line therapy for metastatic melanoma [92]. However, not all patients respond, which is also true for anti-CTLA4 therapy. High mutational load, neoantigaen load and expression of cytolytic markers are beneficial for anti-CTLA4 therapy [93]. In this study, 110 patients were subjected to WES and 40 of those with RNAseq; they observed that high mutational load, neoantigen load, and expression of cytolytic markers were beneficial for response. WES and RNAseq were performed on a set of 38 and 28 but do not have a role in anti-PD1 therapy in melanoma [94]. Mutational load did not have a significant association to response to anti-PD-1 therapy and neoantigen load was not significant for response either. BRCA2 mutations occurred in 30% of the responders to anti-PD-1. However, the frequency of BRCA2 mutations may differ between anti-PD-1 responders and non-responders. Previous studies have shown that tumor mutational and neoantigen load were correlated with response to anti-PD-1 immunotherapy in NSCLC they do seem to have a role in anti-PD1 therapy [95, 96]. Interestingly, resistant tumors expressed a gene signature reminiscent of the invasive phenotype. AXL, ROR2, WNT5A, LOXL2, and TWIST2 were significantly upregulated in the non-responding tumors. The authors also noted that this gene signature is also seen in BRAF inhibitor resistant tumors suggesting an innate biological response to anti-tumor therapy. In another study looking at PD-1 resistance mechanisms, JAK1 and JAK2 mutations were found in two patients who developed new lesions under anti PD-1 treatment. B2M mutations were found in one other patient. JAK1 and JAK2 mutations cause loss of interferon gamma growth induced arrest [97].

During the evolution of a malignancy, there is an intensive interaction with immune cells described as immunoediting [98]. This process can be subdivided into three phases: the elimination phase, the equilibrium, and the escape phase. During the elimination phase, immune cells such as T lymphocytes recognize neoantigens on tumor cells and are able to kill most of them. However, some tumor cells survive and manage to grow, but this growth is controlled by immune response mechanisms [4]. This is called the equilibrium phase. If the tumor cells gain additional mechanisms to suppress immune functions, the tumor cells can escape. We speculate that one of these escape mechanisms is the upregulation of immune suppression factors such as PDL-1 on the surface of tumor cells.

For the immunoediting process, expression of HLA surface molecule is essential and is controlled by interferons. Interestingly, the genomic analysis of acquired resistant tumors during anti-PD-1 therapy showed in two of the four patients resistance-associated alterations of the interferon signal (loss of function mutations in Janus kinase 1 or Janus kinase 2). In addition, a mutation of the Beta-2-microglobulin, which is an essential competent of HLA class 1, was found in another patient [99].

In summary, NGS has provided a wealth of information about the genomic and transcriptomic landscape of melanoma biology and resistance. These studies have shown the genetic and transcriptional events important for melanoma progression as well as resistance to BRAF inhibitors and immunotherapy. Of note is the phenotype switching model, which seems to have an important role in the fundamental behavior of melanoma and in one of the resistance mechanisms to targeted therapy and immunotherapy.

6 *In vitro* tools of melanoma metastasis

While the strength of NGS studies is an unprecedented profiling of large-scale molecular events, finding causal features from high-dimensional data remains a great challenge. Thus, *in vitro* tools such as diverse cell line biobanks that better represent actual tumor diversity [100], more complex but scalable assays that serve as relevant tumor proxies, and *in vivo* models that are translatable to human biology are essential to test the hypotheses generated by NGS projects. Here, we will summarize the latest

in vitro developments that will facilitate the functionalization of genes identified in WES or RNA-seq experiments.

As melanoma is the most aggressive form of skin cancer with a high predisposition to invade and metastasize [101], it is of fundamental importance to select the most appropriate *in vitro* model to study disease progression *ex vivo* [102, 103]. For many years, melanomas have been studied by using cell lines that grow under adherent conditions to form two-dimensional (2D) monolayers. However, 2D cultures do not fully reproduce the tumor three-dimensional (3D) organization, the cell−cell and cell−matrix interactions, and the patchy distribution of oxygen and nutrients. For this reason, 2D monolayers do not represent an adequate system to study the tumor biology and the acquired resistance to therapeutic treatments. In fact, the drug response observed in monolayers often fails to reflect the *in vivo* situation [104, 105]. To date, 3D models have been used in melanoma research for preclinical studies as a compromise between standard 2D cultures and xenografts of human tumors, taking advantage of their reduced costs and time compared to animal models [106].

6.1 Three-dimensional melanoma spheroids

Among 3D tools, melanoma spheroid models mirror the architecture of the tumor and recreate the oxygen/nutrient gradients observed *in vivo* [105]. Comparative gene expression profile studies revealed that genes associated with proliferation, differentiation, resistance to therapy, and migration are differentially expressed in cells maintained as 3D spheroids in comparison with 2D cultures, suggesting the preferential expression of specific constellations of genes in well-defined structures [107−109]. Moreover, the localization of the cells expressing specific markers can be affected by the model used. For example, ERK is mainly found in the growing periphery of spheres, mirroring the sub-compartmental expression of melanoma lesions. On the contrary, it is homogeneously expressed in 2D cultures [110].

Similarly, the choice of *in vitro* models is also relevant in the context of targeted therapy. One of the best examples comes from BRAF inhibitors (BRFAi) such as vemurafenib and dabrafenib. The inhibition of growth and invasive abilities of 3D melanoma spheroids with these drugs recapitulates the results of phase II and III clinical trials.

Melanoma spheroids can be classified into two different groups: (1) multicellular tumor spheroids (MCTSs) and (2) tumorospheres. Although these models share the same 3D structure, they are used for different applications according to their intrinsic features [111]. In contrast to tumorospheres that select a subpopulation of melanoma cells with stemness properties, the multicellular spheroid model (MCTSs) preserves the cell heterogeneity of the tumor [103]. MCTSs can be generated with different protocols, all of them based on the anchorage-independent growth, such as the ʙliquid overlay method,^ the ʙhanging drop method,^ and the encapsulation in alginate-based membranes or with rotating systems [111]. In the liquid overlay method, cells are seeded on plates previously coated with a thin layer of inert material (agarose or polyHEMA) to prevent cell adhesion and promote aggregation. Alternatively, cells can be placed in commercial ultra-low attachment plates. The ʙhanging drop technique^ consists in depositing small drops of cell suspension on a lid to induce the cells to accumulate and give rise to spheroids [112]. MCTSs can also be obtained after encapsulation in microparticles of well-defined and reproducible structure and size, which are compatible with high-throughput screening studies. Finally, MCTSs formation can be induced by using rotating systems (shakers and spinner flasks). Implanting multicellular spheroids into collagen I-coated surfaces allow the study of melanoma migration and invasion. This assay is frequently used to study the metastatic potential of melanoma as it mirrors the intercellular interactions with the tumor microenvironment [113−115]. To further investigate the role of stromal cells in the tumor behavior, MCTSs can be generated from a co-culture of melanoma and different cell types (fibroblasts, endothelial or immune cells).

The second 3D tool to study melanoma biology is the tumorosphere. Unlike MCTSs, their main goal is not to fully replicate the *in vivo* tumor but rather to isolate melanoma cancer stem cells (CSCs). However, whether this model really enriches CSCs is still a matter of debate [116]. The formation of tumorospheres spontaneously occurs when cells are plated at a low density in the presence of serum-free conditioned media (ʙstem cell medium^) supplemented with basic fibroblast and epidermal growth factors. The resulting spherical aggregates derive from the clonal expansion of one single cell [117].

In the recent years, two new protocols have been developed to generate tumor spheroids such as the tissue-derived tumor spheres (TDTSs) and the organotypic multicellular spheroids (OMSs). While TDTSs are generated by partial dissociation of cancer tissues, OMSs are derived from tumor fragments maintained in culture without any dissociation step [118]. To date, these models have been successfully generated only for glioblastoma, lung, bladder, and colorectal cancer, but none of them has been established for melanoma [118].

6.2 Analysis of melanoma spheroids with imaging software and mathematical models

Given the great complexity of the 3D structures and the presence of cells on different focal planes, the analysis of melanospheres is not easy and obvious. For this reason, a huge number of software tools have been developed with the aim to analyze growth, invasion, and drug response. The majority of these protocols allow for the interpretation of the behavior of spheres through the calculation of a set of parameters (diameter, total area, area of invasion, factor shape, percentage of fragmentation, number of invading cells, distance reached in collagen,

etc.), thereby defining a complete picture of the model [119]. To this purpose, spheroids are followed up and pictures are taken at different time points to assess the changes in the tumor morphology and size. Pictures are then converted into binary images and analyzed, manually or through an automated system, with an imaging software (ImageJ, CellProfiler, SpheroidSizer, AnaSp, Image-Pro Analyzer, etc.). In addition, some mathematical models can mirror the distribution and penetration of the drug within spheroids, contributing to highlighting the kinetics and dynamics of the treatment [120]. Although it will be difficult to achieve a general protocol of analysis, its standardization could simplify the complexity of the available mathematical models and encourage industries to consider this as a standard step for the evaluation of drug efficacy.

6.3 Three-dimensional melanoma skin equivalents

One of the critical aspects of melanoma progression is the invasion of the dermal compartment after crossing the basement membrane. The 3D melanoma skin reconstructs represent a better tool to study the mechanisms underlying this early stage of the tumor and to evaluate the toxicity of new therapeutic approaches on the healthy cells of the skin.

Melanoma skin reconstructs are artificial skin consisting of a dermal equivalent, composed of fibroblasts embedded in collagen, and an epidermal compartment, composed of keratinocytes and melanoma cells [105]. This model is representative of the tumor microenvironment and architecture. In addition, melanoma reconstructs accurately recapitulate the different stages of tumor development. It has been demonstrated that melanoma cells are located in the skin equivalents in a different position according to the progression stage from which these cells are derived [121]. In detail, cells from radial growth phase tumors are confined to the epidermis, while those derived from vertical growth phase melanomas are located at the dermal-epidermal junction. This model has been used in the past to evaluate the efficacy of BRAF inhibitors, revealing a decreased proliferation and the induction of apoptosis in melanoma cells [122]. Despite the great utility of skin reconstructs in testing the efficacy and toxicity of new compounds, this model is a time-consuming procedure that requires constant monitoring and more than 15 days to obtain the skin. Moreover, it can be employed only to evaluate the early stages of the tumor, when melanoma cells are still in the dermis. On the other hand, it is the *in vitro* tool that closest resembles the tumor architecture and will undoubtedly play an important role in hypothesis testing from NGS studies prior to the application of even more difficult and costly *in vivo* models.

7 Outlook: new therapeutic strategies

If melanoma progression is thought of in a Darwinistic evolutionary model, this opens the possibility of adapted therapeutic dosage schemes with already available drugs [123]. This might suggest that the goal should be to find an equilibrium between the resistant and sensitive tumor cells to control tumor proliferation. Without therapy, sensitive cells have an advantage over resistant cells, whereas the opposite is true with therapy. By applying drug holiday schemes, in theory, the two populations could then be better controlled [124]. But therapeutic or microenvironmental phenotype switching of cells into slow-cycling, treatment-resistant subclones can add even more complexity to an already complicated phenomenon [48, 125]. Thus, future treatments will have to account for the large variety of resistance patterns that exist, which include innate, adaptive, fixed (i.e., genetic), and non-cell autonomous resistance. Targeting multiple pathways may not only make sense from a resistance perspective but also for controlling both phenotypic states of melanoma cells.

Clinically, tumor regression after termination of MAPK pathway inhibitor treatment is sometimes seen, but overall disease regression or even clinical remission has not been described. Due to tumor heterogeneity and different resistance mechanisms (i.e., both genetic and adaptive) present in an individual patient, not all tumors within one patient will react in the same way upon treatment; hence, it will be very difficult to adjust therapy schemes accordingly. We think future efforts will need to focus on better sampling of tumor material, as one tumor biopsy may not represent a complete tumor or a complete patient. The collection of liquid biopsies (circulating tumor cells) might be an approach that gives a better picture of the heterogeneity within one patient [126]. Likely, combination treatments targeting different pathways or hitting the same pathway twice, or combining therapies with different working mechanisms, will be necessary to induce overall disease regression, after which new therapeutic dosage schemes and strategies can be applied.

There have been unexpectedly great successes in the last decade of translating basic research results into treatment strategies with measureable clinical benefits. But we still need better tools to analyze molecular phenomenon with greater breadth and precision, and also to build better theoretical and *in vitro* models that will more accurately recapitulate the complexity inherent in actual patient tumors. The speed of discovery keeps accelerating, and our efforts to bring that new knowledge to the clinics should keep pace.

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11. Results and Discussion

During the work of this thesis over the past 5 years, research on melanoma worldwide followed on the success of newly FDA approved drugs, which for the first time in history showed an improvement in overall survival for melanoma patients with metastatic disease. However, during these years it became apparent that not all patients respond to the treatments, with immunotherapy especially showing relatively low response rates of approximately 10% for anti-CTLA4 and 33% for anti-PD1 mono-therapy [1, 2], opening the search for biomarkers that could predict treatment benefit. For targeted therapies it became clear that, after an initial response of around 9 months, almost all patients develop resistance to these treatments, which initiated the search for resistance mechanisms.

We also aimed to gain insights in the process of resistance development to targeted MAPK therapy in BRAF or NRAS mutated melanoma. As most experiments for this thesis were planned to be done with in vitro cell cultures, we started with the optimization of generating cell cultures from melanoma biopsy material that we obtained from patients treated in our institution. With standard culturing techniques, only melanoma cell cultures are generated that are proliferative in a cell culture setting and adherent to plastic; however, in order to acquire relevant insights into *in vitro* studies regarding therapeutic resistance, we reasoned that it is essential that the *in vivo* diversity is recapitulated in an *in vitro* setting, as heterogeneity is likely to be an important contributor to resistance development. This optimization resulted in a new protocol for cell culture generation which is successfully integrated in our clinic. At the moment we have a success rate of about 70% in generating cell cultures from native melanoma biopsies, whereas before our success rate was only about 15% [3]. This protocol enabled the establishment of many important cell cultures used in our lab, as well as in the work of our collaborators (Wellbrock et al, University of Manchester), such as the MEK162 resistant NRAS mutated cell culture M130219 and the BRAF inhibitor resistant BRAF mutated cell culture M121224.

We showed that after BRAF inhibitor therapy NRAS mutated cells can be found in resistant tumors [4]. However, this mutation cannot be found in all resistant tumors from the same patients, indicating that different resistance mechanisms exist in one patient [4], which was also observed by other groups [5]. In total, we investigated 6 BRAF mutated patients with an NRAS mutation in their post-BRAF-inhibitor-treatment resistant tumors. None of these patients had an NRAS mutation in their pre-treatment sensitive tumors, suggesting this mutation arose *de novo* during treatment. From the resistant tumors from these 6 patients we generated 7 cell cultures in total. These cell cultures also contained an NRAS mutation in addition to their BRAF mutation, and via single cell clones we could show that BRAF and NRAS mutations are not mutually exclusive, as previously thought, but that they can occur in the same cell. We went on to test the sensitivity of these cell cultures for different inhibitors of the MAPK and PI3K-AKT pathway, and found a different response for every cell culture. Hence we concluded that the presence of a double mutation cannot determine follow-up treatment.

Finally, we focused our efforts on finding resistance mechanisms in NRAS mutated patients treated with a MEK inhibitor. To date, no specific RAS inhibitors are available, and RAS is targeted indirectly by inhibiting essential downstream pathways, such as the MAPK pathway which is the most frequently altered pathway in melanoma. However, the observation that these patients quickly develop resistance on MEK inhibitor treatment, may imply that mutated RAS can quickly adapt and signal through one of its other target pathways, such as the PI3K-AKT pathway and the RalGEF-Ral cascade, among others [6-10]. IRS1 overexpression was found to be a mechanism of BRAF inhibitor resistance in BRAF mutated melanoma and targeting IRS1 with small molecules induced cell death in those tumors [11]. IRS1 is an adapter protein signaling through PI3K and MAPK pathways, therefore we wondered if this would be a possible target for NRAS mutated melanomas as well. In total we tested 18 NRAS mutated cell cultures from our biobank and found that an upregulation of IRS1 expression was associated with MEK162 resistance. However, siRNA mediated IRS1 knockdown in the top 5 high IRS1 expressing cells and IRS1 overexpression experiments in low IRS1 expressing MEK162 sensitive cells showed that IRS1 upregulation was not the cause of the MEK162 resistance. However, we found that the cell culture with the highest IRS1 expression was sensitive to NT157, an IRS1 inhibitor, independent of MEK162 treatment. The mechanism of action of this inhibitor is not by blocking the PI3K-AKT pathway, however, the exact mechanism of action will require further investigation, which is outside the scope of this thesis.

With the current therapies and treatment regimens almost all melanoma patients develop resistance to the treatment. To increase the efficacy of current therapies, several strategies can be proposed in order to postpone or avoid drug resistance.

The recognition that the development of targeted therapy resistance follows some principles of Darwinistic evolution, including selection and competition, suggested that sensitive and resistance cancer cells can be seen as two populations that compete with each other for space and substrate [12]. In the absence of therapy, sensitive cells have an advantage over resistant cells and will populate the patient; whereas in the presence of therapy, resistant cells will become the dominant population. In order to create an equilibrium where the two populations oscillate and none of them will take the upper hand, much like in a well-functioning eco-system where prey and predator are in equilibrium, drugs would need to be administered intermittently; a few weeks in which the drug is given so that the resistant cells are the leading population, followed by a few weeks of drug-holiday in which the sensitive cells can take over. In this way melanoma may even become a chronic disease. For breast cancer, in an *in vivo* murine study the authors used an evolutionary approach for administering paclitaxel; they let the tumors' response on a previous dose determine the timing and amount of the next dose [12]. In this way, the authors were able to control tumor growth, even with no treatment intervals lasting several weeks. A similar result was found in an *in vivo* melanoma xenograft study, were it was found that the resistant tumors become BRAF inhibitor dependent for their growth, and that discontinuation of treatment leads to regression of the resistant population. By treating the mice intermittently with BRAF inhibitors, they were able to postpone development of resistance [13].

In order to overcome resistance, one could also try to specifically target common cellular processes that are different in resistant cells compared to sensitive cells. Upon treatment with a MAPK pathway inhibitor, melanoma cells can enter a slow-cycling drug-tolerant state, characterized by a high expression of the histone demethylase JARID1B and resistance to a wide variety of drugs [14]. By analyzing differences in gene expression, it was found that this slow-cycling state has mitochondrial proteins responsible for oxidative phosphorylation that are upregulated, whereas genes responsible for glycolysis were downregulated. Accordingly, cells overexpressing JARID1B consumed 50% more oxygen than non-overexpressing cells. The authors found that these multi-drug resistant cells could still be targeted by drugs specifically targeting the mitochondrial respiratory chain [14]. Another study found that MAPK inhibitor resistant cell lines had a greater uptake and dependency on glutamine than the sensitive lines, and that these cells could be targeted by drugs targeting glutamine metabolism. Glutamine is an abundant amino-acid in the plasma, and tumors are high consumers of it, as it is an important metabolite for many tasks; it has a role in oxidative stress defense, participates in bioenergetics and it also participates in glucose metabolism.

Another strategy of inhibiting the growth of a heterogeneous pool of melanoma cells with different mutational profiles, including the resistant population, is the creation of an amenable micro-environment for tumor growth. Melanoma cells interact with cells in their micro-environment in order to alter and improve the conditions for tumor growth. Cancer associated fibroblasts have a different transcriptional profile and phenotype compared to normal fibroblasts. By remodeling the extracellular matrix and secreting factors, they aid in tumor growth, angiogenesis, inflammation, metastasis and drug resistance. Zhou et al found that inhibition of b-catenin in fibroblasts prevented them from supporting melanoma tumor growth. Accordingly, melanoma cells grown together with b-catenin knockdown fibroblasts in 3D spheroids had diminished tumor growth and a downregulation of the MAPK pathway [15]. In another study, where the authors pharmacologically inhibited fibroblasts activation protein (FAP) on fibroblasts in mice, they could show an attenuated growth of lung cancer and colon cancer cells *in vivo* [16]. In 2015, Jackson et al. published the development of specific inhibitors for FAP and prolyl oligopeptidase (POP) [17]. They could show that these inhibitors reduced growth of colon cancer xenografts on mice by more than 90%, and at immunohistochemistry, the treated tumors had fewer microvessels [17]. In an older study from 2005, the authors immunized mice against FAP and could show that they had diminished growth of implanted melanoma tumors [18]. The tumor micro-environment also harbors other cells such as immune cells and endothelial cells, which can be altered in order to become harmless (immune cells) or even supportive for melanoma growth. The successful anti-PD1 therapy works by blocking the immune-inhibitory signal on melanoma cells, thereby activating the immune system to eradicate the melanoma cells.

As resistance is often mediated by signaling through escape pathways, for example the PI3K pathway in MAPK inhibitor resistant melanoma, one could also think of combining drugs targeting different pathways at the same time. However, precaution is warranted with such an approach, as certain pathways may influence and antagonize each other. Another possibility may be to sequentially treat with different pathway inhibitors, depending on the resistance mechanism that patient develops, which is an approach termed precision medicine. However, as it has been shown by us and others that different metastasis in the same patient can have different resistance mechanisms, current trials need to show whether this approach will lead to major benefits.

It is expected that targeted therapy will remain important in melanoma treatment, despite the lack of durable effects, such as seen in a small patient population treated with immunotherapy. However, targeted therapy has some important advantages over immunotherapy, such as a higher and faster response rate and fewer side-effects, which is especially important for patients who are in a bad condition due to their illness. Therefore, it is essential to try to improve this treatment modality of melanoma. Improvements have to be made to extend the time of treatment response before resistance develops and to develop more targeted therapies for prevalent melanoma mutations/pathway alterations, other than BRAF.

We will continue to investigate MAPK pathway resistance mechanisms in NRAS and BRAF mutated melanoma, in order to anticipate on and thereby overcome or delay therapy resistance. NRAS mutated melanoma patients are especially in need of alternative treatment options, as these patients quickly develop resistance to MEK inhibitors, which are to date the only targeted therapy option for this patient group. In the last decade, research has mainly focused on the MAPK pathway, but as NRAS can also signal via alternative pathways, it will be interesting to look for other possible targetable pathways.

Advances can also be made with new techniques for studying tumor biology such as in *in vitro* cell cultures, including the 3D culture systems such as growing melanoma cells in 3D spheroids, or culturing them together with fibroblasts and keratinocytes, for instance in an *in vitro* artificial skin construct. For certain research questions, including drug resistance, these systems are thought to better represent the in vivo situation and thus can give additional information about melanoma versus melanoma and melanoma versus micro-environment interactions. It would be interesting to check new possible therapies, including the IRS1 inhibitor NT157, in these culture systems.

With the availability of next generation sequencing techniques, there is an increased interest in personalized targeted therapy selection based on RNA and DNA sequencing. So far however, results have been disappointing, probably due to limitations of only analyzing single biospies, whereas melanoma has high intra-patient heterogeneity. As we have, for some patients, material from multiple tumor sites available in our biobank, we have the possibility to first test this concept in an *in vitro* setting. Especially when combined with 3D *in vitro* techniques and/or co-cultures from different melanoma cells from the same patient, this might give valuable results whether such an approach of personalized medicine is beneficial. This also opens the possibility of identifying rational combinations of different inhibitors and examine their combined effect *in vitro*.

A large amount of progress has been made over the last years in treating metastatic melanoma, however, a lot still needs to be done before it can be cured or kept under control for every patient. Continuing world-wide efforts to understand melanoma biology, its resistance mechanisms during therapy, and evolution during disease progression are the best ways to reach that goal.

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Curriculum Vitae

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Education:

1998 – 2004	Secondary School, Walburgcollege, Zwijndrecht, the Netherlands Matura type Atheneum, Profile "Nature and Technique" and "Nature and Health"
2004 – 2011	Medicine, Erasmus University Rotterdam, the Netherlands
2006 – 2010	Research Master of Science "Molecular Medicine", Erasmus University Rotterdam, the Netherlands Master Thesis: "Role of activin A and the novel activin variant activin E in pathologic arterial wall calcification"
Since 2012	Employed as a MD PhD student, University of Zürich, Switzerland PhD Thesis: "On the origin of melanoma targeted therapy resistance"

Presentations during the doctoral studies:

Poster presentations	42nd European Society for Dermatological Research 2012, Vienna, Italy
	17th European Society for Pigment Cell Research conference 2012, Geneva, Switzerland
	5th Cancer Network Zürich Retreat 2013, Grindelwald, Switzerland
	Zürich/Basel MD PhD Retreat 2013, Ascona, Switzerland
	11th International Congres of the Society for Melanoma Research 2014, Zürich, Switzerland
	12th Charles Rudolphe Brupbacher Symposium 2015, Zürich, Switzerland
	15th Day of Clinical Research 2016, University of Zürich, Switzerland
Oral presentations	SSS Meeting Davos, Switzerland, retreat from department of Dermatology, University Hospital Zürich in 2012, 2014 and 2015
	Fortschritte in der dermatologischen and allergologischen Forschung (Vorlesungsnummer 1205), University of Zürich, 2012, 2013, 2014, 2015 and 2016
	Joint Cancer Meeting, Cancer Network Zürich, 2016
	Wagi Lunch Seminar Series, University of Zürich, Schlieren, 2016

Publications:

1. **Raaijmakers, M.I.**, D.S. Widmer, M. Maudrich, T. Koch, A. Langer, A. Flace, C. Schnyder, R. Dummer, and M.P. Levesque, *A new live-cell biobank workflow efficiently recovers heterogeneous melanoma cells from native biopsies.* Exp Dermatol, 2015. **24**(5): p. 377-80.
2. **Raaijmakers, M.I.**, D.S. Widmer, A. Narechania, O. Eichhoff, S.N. Freiberger, J. Wenzina, P.F. Cheng, D. Mihic-Probst, R. Desalle, R. Dummer, and M.P. Levesque, *Co-existence of BRAF and NRAS driver mutations in the same melanoma cells results in heterogeneity of targeted therapy resistance.* Oncotarget, 2016. **7**(47): p. 77163-77174.

3. **Raaijmakers, M.I.**, S. Rozati, S.M. Goldinger, D.S. Widmer, R. Dummer, and M.P. Levesque, *Melanoma immunotherapy: historical precedents, recent successes and future prospects.* Immunotherapy, 2013. **5**(2): p. 169-82.
4. Levesque, M.P., P.F. Cheng, **M.I. Raaijmakers**, A. Saltari, and R. Dummer, *Metastatic melanoma moves on: translational science in the era of personalized medicine.* Cancer Metastasis Rev, 2017.
5. Widmer, D.S., K.S. Hoek, P.F. Cheng, O.M. Eichhoff, T. Biedermann, **M.I. Raaijmakers**, S. Hemmi, R. Dummer, and M.P. Levesque, *Hypoxia contributes to melanoma heterogeneity by triggering HIF1alpha-dependent phenotype switching.* J Invest Dermatol, 2013. **133**(10): p. 2436-43.
6. Cheng, P.F., O. Shakhova, D.S. Widmer, O.M. Eichhoff, D. Zingg, S.C. Frommel, B. Belloni, **M.I. Raaijmakers**, S.M. Goldinger, R. Santoro, S. Hemmi, L. Sommer, R. Dummer, and M.P. Levesque, *Methylation-dependent SOX9 expression mediates invasion in human melanoma cells and is a negative prognostic factor in advanced melanoma.* Genome Biol, 2015. **16**: p. 42.

Other activities:

2014	Organization 3rd European MD PhD Retreat in Brunnen, Switzerland
2006 – 2013	Editorial Assistant for international peer-reviewed journal “Strabismus”, department of Ophthalmology, Erasmus Medical Center, Rotterdam, the Netherlands